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# PROCEEDINGS

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PROCEEDINGS OF

THE ROYAL SOCIETY.

*Section B.—Biological Sciences.*

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# *The Effect of Plant Growth and of Manures upon the Retention of Bases by the Soil.*

By A. D. Hall, M.A., and N. H. J. Miller, Ph.D.

(Communicated by H. E. Armstrong, LL.D., Ph.D., F.R.S. (From the Lawes Agricultural Trust.) Received March 30,—Read May 11, 1905.)

## *Introductory.*

The following investigation deals with the changes in the amount of calcium carbonate, the chief substance in the soil acting as a base, which are brought about by natural agencies, by manuring, and particularly by the growth of plants.

Since Cavendish discovered that calcium carbonate dissolves in water charged with carbon dioxide, and ascertained the presence of calcium bicarbonate in many natural waters, it has been recognised that the calcium carbonate present in most soils must be subject to regular loss. The air enclosed in the soil contains a considerable proportion of carbon dioxide derived from the roots of plants and decaying organic matter (Wollny, for example, found amounts varying from 3 to 14 per cent. by volume), and the soil water, after attaining a state of equilibrium with the gas at this partial pressure, becomes an effective solvent of any calcium carbonate that may be present, removing it as bicarbonate into the drains or the general stock of underground water. That such dissolution does take place in the upper layers of the soil is evident from the analyses made by A. Voelcker* and

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* 'Journ. Chem. Soc.,' 1871, p. 276.

Frankland* of the drainage waters from the Broadbalk wheatfield at Rothamsted.† Although the tile drains, from the flow of which the waters in question are derived, rarely lie more than 2 feet 6 inches below the surface of the soil, yet the drainage water from the unmanured plot contained on the average 99 parts per million of CaO, of which 84 were in the state of bicarbonate, and the water from the plot receiving farmyard manure every year contained 147 parts of CaO per million, of which 72 may be regarded as in the state of bicarbonate. Such concentrations, representing a hardness of about 17 and 26 degrees respectively, though by no means equal to those of truly calcareous waters, are above the average of natural waters in this country; yet, as will be seen later, the calcium carbonate from which they are derived is practically confined to the uppermost 9 inches of soil.

While such losses may be regarded as natural, it has long been known that many of the substances applied to the soil under the general term of artificial manures react with the calcium carbonate there present and bring about its dissolution. Liebig, for example, pointed out that the di-hydrogen calcium phosphate ("superphosphate") contained in bones made soluble by treatment with sulphuric acid, reacts at once with the bases in the soil and becomes again insoluble. The researches of Way‡ and A. Voelcker§ showed further that the retention of ammonium and potassium salts by cultivated soils is always preceded by a double decomposition with calcium carbonate, the bases being retained as carbonates while the acids appear in the drainage waters combined with calcium. A. Voelcker's analyses of the Rothamsted drainage waters,|| when dealing with the plots receiving salts of ammonium, potassium, etc., as manures, show the same reactions taking place on a large scale.

Again, the process of nitrification, going on in all normal soils, requires some base to combine with the nitrous and nitric acids produced by the oxidation of the ammonia and other nitrogen compounds.¶ In an ordinary way this base is supplied by calcium carbonate, hence a further source of loss to the calcium carbonate of cultivated soils.

The soils of the Rothamsted experimental plots afford peculiar facilities for the study of the rate at which these losses of calcium carbonate, both natural

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* 'Journ. Roy. Agri. Soc.,' 2nd Series, vol. 18, 1882, p. 14.

† See Table X.

‡ 'Journ. Roy. Agri. Soc.,' 1st Series, vol. 11, 1850, p. 313, and vol. 13, 1852, p. 123.

§ 'Journ. Roy. Agri. Soc.,' 1st Series, vol. 21, 1860, p. 105, and vol. 25, 1864, p. 333.

|| *Loc. cit.*

¶ 'Instruction sur l'établissement des Nitrières,' Paris, 1777; Warington, 'Trans. Chem. Soc.,' 1879, p. 429.

and induced by the application of saline manures, are taking place. In most cases the plots have received the same manures, year after year, for more than fifty years, and though unfortunately samples of the soil were not taken at the starting of the experiments, yet in the case of the Broadbalk Field, on which wheat has been grown continuously since 1843, a set of samples drawn in 1856 has been preserved, in addition to samples drawn in 1865 and in 1893. From the Hoos Field (continuous barley since 1852) samples were drawn in 1868, 1882, and 1904—5, and from Agdell Field (under a four-course rotation since 1848) samples exist which were drawn in 1867, 1874, 1883, and 1905.

Furthermore, the calcium carbonate in the Rothamsted soil is of extraneous origin, and is entirely localised in the surface layer which is stirred by the plough. The subsoil, from which the surface soil is undoubtedly derived, belongs to the drift formation of "clay-with-flints," characteristic of the chalk plateau, and consists of the *débris* of the chalk formation largely mixed with sands and clays of the Reading series.* It normally contains little or no calcium carbonate, although it is partly derived from the chalk formation and rests upon the solid chalk at a varying depth of 8 to 12 or 20 feet.

In the eighteenth century, however, a characteristic feature of the agriculture of this district of Hertfordshire was to manure the land by sinking pits through the clay to the chalk, which was then lifted and spread in considerable quantities. Arthur Young† quotes from Walker's Survey of 1795—"the now prevailing practice of sinking pits for the purpose of chalking the surrounding land therefrom . . . The most experienced Hertfordshire farmers agree that chalking of lands so circumstanced is the best mode of culture they are capable of receiving." Evidence of the former prevalence of this practice of chalking may be seen by the existence in each of the Rothamsted fields of a "dell," a depression representing the fallen-in pit from which the chalk was extracted. A certain rawness of the soil round the edges of these "dells" still bears witness to the disturbance created by the excavation, though it is known that nothing of the kind was done during the late Sir John Lawes' possession of the estate, which dates back to 1834. Probably the pits were but little worked after the close of the eighteenth century, and certainly neither chalk nor lime has been applied to the plots since they were put under experiment. At the present time the chalk is visible only in the upper soil, and is there present in small rounded nodules varying in diameter from 3 or 4 mm. downwards.

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* See H. B. Woodward, "Report of the Soils and Subsoils of the Rothamsted Estate. Summary of Progress of the Geological Survey, 1903."

† "Report on the Present State of the Agriculture of Hertfordshire," presented to the Board of Agriculture, 1804.

## I.—CALCIUM CARBONATE IN ROTHAMSTED SOILS.

### A. *Analytical.*

The first section of this paper deals with the determinations of the amounts of calcium carbonate present in the soils and subsoils of certain of the plots in the Broadbalk, Hoos, Agdell, and Little Hoos Fields, the samples having been drawn at the dates specified above and again in 1904—5. As the calcium carbonate is of artificial origin, and was probably distributed with considerable irregularity, however much this may have been equalised by the subsequent working to which the soil has been subjected, it cannot be expected that the samples analysed will represent the whole soil of the plots with the same degree of accuracy as would be attained in the case of some original constituent of the soil.

Arable soils only are considered; with soils in permanent grass the question is complicated by the well-known action of earthworms, which, as demonstrated by Darwin, bury the surface layer by constantly bringing fine subsoil to the top.

The determinations of calcium carbonate have been made by means of an apparatus described by one of us in conjunction with Dr. E. J. Russell.* The results are calculated from the volume of carbon dioxide evolved on treating the fine soil with dilute sulphuric acid *in vacuo*, due provision being made to bring into account the carbon dioxide remaining dissolved in the reacting liquid. In a few cases, where the percentage of calcium carbonate was very low, the carbon dioxide evolved by treating a considerable quantity of the soil with acid was absorbed by caustic soda and determined by double titration. For these determinations we have to thank Mr. Arthur Amos, B.A. The amount of magnesium carbonate present is too small to affect the results, and in any case, as the real quantity sought is the amount of readily available base in the soil, it is desirable to express it always in the same terms.

The soil samples were all taken in the same way: a steel frame 6 inches or 1 foot square and 9 inches deep is driven into the ground, and its contents are carefully picked out; this gives the soil proper. The surrounding soil is then dug away and the frame is driven down another 9 inches. The contents now represent the subsoil at the second depth of 10 to 18 inches. The process is then repeated to as many holes as may be required. In this way samples are taken from four, six, or eight holes on each plot, according to its size.

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* Hall and Russell, 'Trans. Chem. Soc.,' vol. 81, 1902, p. 145.

The samples, after drying at a temperature not exceeding 60° C., are roughly powdered and put through a woven wire sieve with a mesh of $\frac{1}{4}$ inch (this sieve passes a little more than the 3 mm. round-hole sieve now commonly used). From the fine earth thus obtained from each hole composite samples representing the whole plot at each depth are then made up, of which portions are finely ground for analysis.

Table I gives details of the manurial treatment of the various plots in Broadbalk and Hoos Fields, in the soil of which the calcium carbonate has been determined; Table II gives the average weight in pounds per acre of the fine dry soil in the layer 9 inches deep which is removed by the sampling tool for the different fields at each of the specified dates; Table III for Broadbalk, Table V for Hoos, and Table VII for Agdell and Little Hoos Fields give the percentages of calcium carbonate in the fine soils dried at 100° C.

Table I.—Nature and Quantities per Acre of the Manures annually applied.*

| Plot. | Date of beginning treatment. | Farmyard manure. | Sodium nitrate. | Ammonium sulphate. | Ammonium chloride. | Rape cake. | Super-phosphate. | Potassium sulphate. | Sodium sulphate. | Magnesium sulphate. |
|---|---|---|---|---|---|---|---|---|---|---|
| Broadbalk Field. | | | | | | | | | | |
| | | tons. | lbs. | lbs. | lbs. | lbs. | cwts. | lbs. | lbs. | lbs. |
| 3 | 1844 | Unmanured | — | — | — | — | — | — | — | — |
| 2B | 1844 | 14 | — | — | — | — | — | — | — | — |
| 5 | 1852 | — | — | — | — | — | 3·5 | 200 | 100 | 100 |
| 6 | 1852 | — | — | 100 | 100 | — | 3·5 | 200 | 100 | 100 |
| 7 | 1852 | — | — | 200 | 200 | — | 3·5 | 200 | 100 | 100 |
| 8 | 1852 | — | — | 300 | 300 | — | 3·5 | 200 | 100 | 100 |
| 9 | 1852 | — | 275† | — | — | — | 3·5 | 200 | 100 | 100 |
| 10 | 1845 | — | — | 200 | 200 | — | — | — | — | — |
| 11 | 1849 | — | — | 200 | 200 | — | 3·5 | — | — | — |
| Hoos Field. | | | | | | | | | | |
| 1o | 1852 | Unmanured | — | — | — | — | — | — | — | — |
| 4o | 1852 | — | — | — | — | — | 3·5 | 200 | 100 | 100 |
| 1A | 1852 | — | — | 100 | 100 | — | — | — | — | — |
| 4A | 1852 | — | — | 100 | 100 | — | 3·5 | 200 | 100 | 100 |
| 1N | 1868 | — | 275 | — | — | — | — | — | — | — |
| 4N | 1868 | — | 275 | — | — | — | 3·5 | 200 | 100 | 100 |
| 1c | 1852 | — | — | — | — | 1000 | — | — | — | — |
| 7—2 | 1852 | 14 | — | — | — | — | — | — | — | — |

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* For certain minor variations in the amounts of manure applied see "Memoranda of the Field and other Experiments at Rothamsted. Lawes Agricultural Trust, 1901."

† 550 lbs. per annum up to 1884.

Certain difficulties are experienced in attempting to calculate from these figures the actual quantity of calcium carbonate per acre in the soil at different periods, owing to the impossibility of drawing samples that represent the same layer of soil on each occasion. Since changes of texture are set up by the different treatment of the plots the consolidation of the surface layer varies, so that the 9-inch slice includes more or less soil from time to time. On most plots the weights of the samples tend to get heavier, because the soil sets more closely together under the conditions of long-continued manuring

Table II.—Weights of Fine Soil Dried at 100° C. per Acre.

Broadbalk Field.

| Plots. | Depth. | 1856. | 1865. | 1881. | 1893. | 1904. |
|---|---|---|---|---|---|---|
| | | lbs. | lbs. | lbs. | lbs. | lbs. |
| 2*b* | 1st 9 inches | — | 2,200,000 | 2,400,000 | 2,400,000 | 2,400,000 |
| Other plots | 1st 9 inches | 2,200,000 | 2,300,000 | 2,560,000 | 2,650,000 | 2,650,000 |
| All plots | 2nd 9 inches | 2,590,000 | 2,590,000 | 2,590,000 | 2,590,000 | 2,590,000 |
| All plots | 3rd 9 inches | 2,815,000 | 2,815,000 | 2,815,000 | 2,815,000 | 2,815,000 |

Hoos Field.

| Plots. | Depth. | 1868. | 1882. | 1904–5. |
|---|---|---|---|---|
| | | lbs. | lbs. | lbs. |
| 7–2 | 1st 9 inches | — | 2,100,000 | 2,100,000 |
| Other plots | 1st 9 inches | 2,400,000 | 2,400,000 | 2,400,000 |
| All plots | 2nd 9 inches | 2,721,000 | 2.721,000 | 2,721,000 |
| All plots | 3rd 9 inches | 2,891,000 | 2,891,000 | 2,891,000 |

Agdell Field.

| Plots. | Depth. | 1867. | 1874. | 1883–4. | 1905. |
|---|---|---|---|---|---|
| | | lbs. | lbs. | lbs. | lbs. |
| All plots | 1st 9 inches | 2,140,000 | 2,400,000 | 2,500,000 | 2,500,000 |
| All plots | 2nd 9 inches | 2,450,000 | 2,450,000 | 2,450,900 | 2,450,000 |

Little Hoos Field.

| Plots. | Depth. | 1873. | 1904. |
|---|---|---|---|
| | | lbs. | lbs. |
| All plots | 1st 9 inches | 2,500,000 | 2,500,000 |
| All plots | 2nd 9 inches | 2,500,000 | 2,500,000 |

Table III.—Broadbalk Wheat Soils—collected at various dates.

*Calcium Carbonate* per cent. in Fine Soil Dried at 100° C.

| Plot. | Manures. | Sept., 1856. | Oct., 1865. | Oct., 1881. | Oct., 1893. | Sept., 1904. |
|---|---|---|---|---|---|---|
| | 1st Depth (1—9 inches). | | | | | |
| | | p. c. | p. c. | p. c. | p. c. | p. c. |
| 2*b* | Farmyard manure (14 tons) | — | 4·20 | 3·79 | 3·46 | 3·28 |
| 3 | Unmanured | 5·35 | 4·54 | 3·97 | 3·45 | 3·29 |
| 5 | Full minerals | 5·65 | 4·96 | 3·75 | 3·34 | 2·94 |
| 6 | „ + 200 lbs. ammonium salts | — | — | 3·41 | 1·98 | 2·33 |
| 7 | „ + 400 lbs. „ | — | 3·82 | 3·19 | 2·36 | 2·25 |
| 8 | „ + 600 lbs. „ | — | — | 2·84 | 1·73 | 1·76 |
| 9 | „ + 275* lbs. nitrate soda | — | 4·24 | 3·99 | 3·72 | 3·36 |
| 10 | 400 lbs. ammonium salts only | 5·41 | 4·10 | 3·31 | 2·76 | 2·47 |
| 11 | „ „ and superphosphate | — | 4·36 | 3·14 | 2·76 | — |
| | 2nd Depth (10—18 inches). | | | | | |
| 2*b* | Farmyard manure (14 tons) | — | 0·277 | 0·310 | 0·422 | 0·237 |
| 3 | Unmanured | — | 0·222 | 0·162 | 0·099 | 0·116 |
| 5 | Full minerals | — | 0·147 | 0·128 | 0·100 | 0·110 |
| 6 | „ + 200 lbs. ammonium salts | — | — | 0·110 | 0·114 | 0·132 |
| 7 | „ + 400 lbs. „ | — | 0·212 | 0·210 | 0·096 | 0·167 |
| 8 | „ + 600 lbs. „ | — | — | 0·106 | 0·093 | 0·117 |
| 9 | „ + 275* lbs. nitrate soda | — | 0·309 | 0·263 | 0·482 | 0·143 |
| 10 | 400 lbs. ammonium salts only | — | 0·127 | 0·137 | 0·170 | 0·111 |
| 11 | „ „ and superphosphate | — | 0·119 | 0·179 | 0·107 | — |
| | 3rd Depth (19—27 inches). | | | | | |
| 2*b* | Farmyard manure (14 tons) | — | 0·181 | 0·121 | 0·130 | 0·095 |
| 3 | Unmanured | — | 0·179 | 0·090 | 0·084 | 0·113 |
| 5 | Full minerals | — | 0·056 | 0·079 | 0·050 | 0·100 |
| 6 | „ + 200 lbs. ammonium salts | — | — | 0·058 | 0·073 | 0·112 |
| 7 | „ + 400 lbs. „ | — | 0·144 | 0·115 | 0·075 | 0·136 |
| 8 | „ + 600 lbs. „ | — | — | 0·063 | 0·104 | 0·122 |
| 9 | „ + 275* lbs. nitrate soda | — | 0·144 | 0·102 | 0·130 | 0·116 |
| 10 | 400 lbs. ammonium salts only | — | 0·073 | 0·070 | 0·070 | 0·105 |
| 11 | „ „ and superphosphate | — | 0·090 | 0·089 | 0·085 | — |

* Double this amount applied from 1855 to 1884 inclusive.

with saline manures and the gradual loss of organic matter. On the contrary, the soil of the plot receiving farmyard manure grows lighter through the great accumulation of organic matter. These changes of weight would be of little moment were the soil uniform, but as the calcium carbonate is almost wholly present in the surface soil down to about six or seven inches, small variations in the thickness of the slice taken by the tool cause a varying admixture of the poorer subsoil, and so may induce considerable change in the estimated weights of calcium carbonate. It is impossible to eliminate wholly the errors thus introduced, especially in the case of Broadbalk, where the average weights of the 1865 samples are exceptionally low and where the subsoil shows also a good deal of variation.

In Table IV two estimates have been drawn up for the amounts of calcium carbonate per acre. In the upper set of figures an attempt has been made to introduce a correction for the varying thickness of the slice by adding to the weights of calcium carbonate in the first and second depths such a proportion of the third depth as would ensure the comparison of an equal weight of soil in all cases. The lower set of figures is based simply upon the percentage of calcium carbonate in the upper layer of soil, assuming a general

Table IV.—Broadbalk Soils.

*Calcium Carbonate* in Lbs. per Acre.

| Plot. | 1865. | 1881. | 1893. | 1904. | Rate of loss per acre per annum. | |
|---|---|---|---|---|---|---|
| | | | | | Whole period. | 1881—1904. |
| Total of 1st and 2nd 9 Inches + a fraction of the 3rd 9 Inches, to bring them all to the same Total of 5,240,000 Lbs. of Soil per Acre. | | | | | | |
| | lbs. | lbs. | lbs. | lbs. | lbs. | lbs. |
| 2*b* | 100,400 | 99,300 | 94,300 | 85,100 | 392 | 618 |
| 3 | 110,800 | 105,900 | 94,000 | 90,200 | 528 | 683 |
| 5 | 118,100 | 99,400 | 91,100 | 80,800 | 809* | 809 |
| 6 | — | 90,200 | 55,400 | 65,200 | 1086 | 1086 |
| 7 | 93,900 | 87,200 | 65,000 | 64,000 | 767 | 1009 |
| 8 | — | 75,500 | 48,300 | 49,700 | 1122 | 1122 |
| 9 | 106,000 | 109,100 | 111,100 | 92,700 | 341 | 713 |
| 10 | 97,800 | 88,400 | 77,500 | 68,300 | 756 | 874 |
| 11 | 103,700 | 85,100 | 75,900 | — | 993 | — |
| First 9 Inches only, reckoned as weighing 2,500,000 Lbs. | | | | | | |
| 2*b* | 105,000 | 94,700 | 86,500 | 82,000 | 590 | 552 |
| 3 | 113,500 | 99,200 | 86,200 | 82,200 | 800 | 739 |
| 5 | 124,000 | 93,700 | 83,500 | 73,500 | 878* | 878 |
| 6 | — | 85,200 | 49,500 | 58,200 | 1174 | 1174 |
| 7 | 95,500 | 79,750 | 59,000 | 56,200 | 1010 | 1024 |
| 8 | — | 71,000 | 43,200 | 44,000 | 1174 | 1174 |
| 9 | 106,000 | 99,700 | 93,000 | 84,000 | 564 | 683 |
| 10 | 102,500 | 82,700 | 69,000 | 61,700 | 1045 | 913 |
| 11 | 109,000 | 78,500 | 69,000 | — | 1429 | — |

* 1881—1904 only.

average weight of 2,500,000 lbs. for the fine dry earth in the top 9 inches of soil, thus leaving out of account both the changing weights of the upper slice and the contents of the subsoils. The two sets of figures lead to much the same comparative results, but the lower table is to be preferred as free from any speculative corrections, bearing in mind, however, that the rate of loss is probably over-estimated on most of the plots and under-estimated on the plot receiving farmyard manure. Fig. 1 shows these percentages of calcium carbonate in the upper soil plotted against the time.

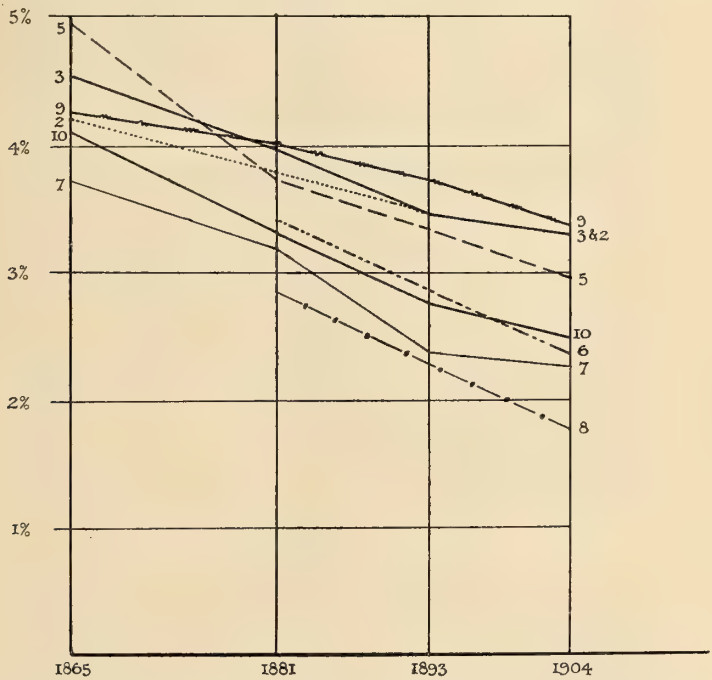

Fig. 1.—Percentage of Calcium Carbonate. Broadbalk Field. First 9 inches.

A cursory examination of the figures and curves shows that if it may be assumed that the calcium carbonate was equally distributed over the whole field initially, then the greatest losses have occurred on the plots manured with ammonium salts, the loss increasing with each addition of ammonium salts; sodium nitrate, on the contrary, would seem to have exercised some preservative influence on the calcium carbonate, which is now at its maximum on the plot where that manure is used.

Table VI gives the weights of calcium carbonate per acre in soils from the Hoos field as calculated from the percentages in Table V and from the average weights of soil in Table II, without any attempt at correction; the results being also thrown into a graphic form in fig. 2.

Table V.—Hoos Field Barley Soils, collected at various dates.

*Calcium Carbonate* per cent. in Fine Soil dried at 100° C.

| Plot. | Manures. | 1868. | 1882. | 1904–5. |
|---|---|---|---|---|
| | 1st Depth (1—9 inches). | | | |
| | | Per cent. | Per cent. | Per cent. |
| 1O | Unmanured | — | 3·43 | 2·34 |
| 4O | Full minerals | 1·78 | 1·47 | 0·71 |
| 1A | 200 lbs. ammonium salts | — | 3·26 | 2·53 |
| 4A | Full minerals + 200 lbs. ammonium salts | 1·34 | 0·87 | 0·23 |
| 1N | 275 lbs. nitrate of soda | — | 3·30 | 2·59 |
| 4N | Full minerals + 275 lbs. nitrate of soda | 1·30 | 1·32 | 0·48 |
| 1C | 1000 lbs. rape cake | — | 1·58 | 0·89 |
| 7—2 | 14 tons farmyard manure | — | 1·92 | 1·14 |
| | 2nd Depth (10—18 inches). | | | |
| 1O | Unmanured | — | 0·107 | 0·202 |
| 4O | Full minerals | 0·110 | 0·091 | 0·139 |
| 1A | 200 lbs. ammonium salts | — | 0·116 | 0·119 |
| 4A | Full minerals + 200 lbs. ammonium salts | 0·154 | 0·074 | 0·083 |
| 1N | 275 lbs. nitrate of soda | — | 0·107 | 0·112 |
| 4N | Full minerals + 275 lbs. nitrate of soda | 0·064 | 0·073 | 0·112 |
| 1C | 1000 lbs. rape cake | — | 0·095 | 0·066 |
| 7—2 | 14 tons farmyard manure | — | 0·275 | 0·147 |
| | 3rd Depth (19—27 inches). | | | |
| 1O | Unmanured | — | 0·056 | 0·078 |
| 4O | Full minerals | 0·094 | 0·081 | 0·091 |
| 1A | 200 lbs. ammonium salts | — | 0·063 | 0·062 |
| 4A | Full minerals + 200 lbs. ammonium salts | 0·096 | 0·090 | 0·073 |
| 1N | 275 lbs. nitrate of soda | — | 0·075 | 0·084 |
| 4N | Full minerals + 275 lbs. nitrate of soda | 0·063 | 0·061 | 0·103 |
| 1C | 1000 lbs. rape cake | — | 0·068 | 0·067 |
| 7—2 | 14 tons farmyard manure | — | 0·074 | 0·103 |

Unfortunately the figures show at once that the initial chalking of the Hoos Field has been very irregular, the plots numbered 1 show more than twice as much calcium carbonate as the corresponding plots numbered 4. As also in this field, samples only exist for two dates, 1882 and 1904—5, instead of the four which are available for the Broadbalk Field, less weight can be laid on the results for the individual plots. It is, however, noticeable that it is one of the plots receiving ammonium salts every year which now contains the least calcium carbonate.

Table VI.—Hoos Field Soils.

*Calcium Carbonate* in Lbs. per Acre.

| Plot. | 1868. | 1882. | 1904–5. | Rate of loss per acre per annum. |
|---|---|---|---|---|
| Total for Three Depths (27 inches). | | | | |
| | lbs. | lbs. | lbs. | lbs. |
| 1O | — | 86,800 | 63,900 | 1000 |
| 4O | 48,431 | 40,100 | 23,400 | 675 |
| 1A | — | 83,200 | 65,700 | 760 |
| 4A | 39,100 | 25,500 | 9,900 | 790 |
| 1N | — | 84,300 | 67,600 | 725 |
| 4N | 34,754 | 35,400 | 17,500 | 465 |
| 1C | — | 42,500 | 25,100 | 755 |
| 7—2 | — | 49,900 | 30,900 | 830 |
| First 9 inches only, reckoned as weighing 2,500,000 lbs. | | | | |
| 1O | — | 85,750 | 58,500 | 1185 |
| 4O | 44,500 | 36,750 | 17,750 | 723 |
| 1A | — | 81,500 | 63,250 | 793 |
| 4A | 33,500 | 21,750 | 5,750 | 750 |
| 1N | — | 82,500 | 64,750 | 772 |
| 4N | 32,500 | 33,000 | 12,000 | 554 |
| 1C | — | 39,500 | 22,250 | 750 |
| 7—2 | — | 48,000 | 28,500 | 848 |

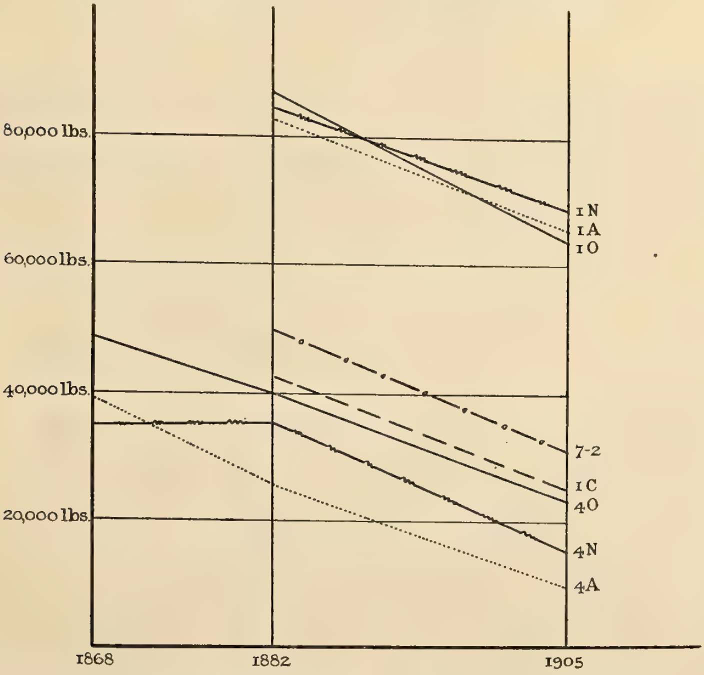

Fig. 2.—Calcium Carbonate, lbs. per acre. Hoos Field. To 27 inches.

Table VII gives the percentages of calcium carbonate found in the soils of the Agdell and the Little Hoos Fields, which are not specifically manured.

Table VII.—Calcium Carbonate per cent. in Fine Soil dried at 100° C.

Agdell Field.

| | 1867. | 1874. | 1883–4. | 1905. |
|---|---|---|---|---|
| 1st Depth (1—9 inches). | | | | |
| Unmanured | 5·815 | 5·984 | 5·102 | 4·522 |
| Unmanured | 5·912 | 6·414 | 4·927 | 4·567 |
| 2nd Depth (10—18 inches). | | | | |
| Unmanured | 0·304 | 0·514 | 0·137 | 0·195 |
| Unmanured | 0·220 | 0·767 | 0·134 | 0·138 |

Little Hoos Field.

| | 1873. | 1904. |
|---|---|---|
| 1st 9 inches | 3·974 | 2·704 |
| 2nd 9 inches | 0·145 | 0·118 |

Lastly in Table VIII the rates of loss in all cases are brought together for comparison. In calculating these rates of loss the earliest samples (1856) from the Broadbalk Field have been rejected as not comparable with the

Table VIII.—Rate of Loss of Calcium Carbonate from Soil, lbs. per Acre per Annum.

| | Broadbalk wheat. | Hoos barley. | Agdell rotation. | Little Hoos, various. |
|---|---|---|---|---|
| Unmanured | 800 | 1000 | 922<br>938 | 1046 |
| Mineral manures only | 880 | 675 | | |
| ,, + 200 lbs. ammonium salts | 1170 | 790 | | |
| ,, + 400 lbs. ,, ,, | 1010 | — | | |
| ,, + 600 lbs. ,, ,, | 1170 | — | | |
| ,, + 275 lbs. sodium nitrate | 565 | 465 | | |
| 200 lbs. ammonium salts only | — | 760 | | |
| 400 lbs. ,, ,, | 1045 | — | | |
| 275 lbs. sodium nitrate only | — | 725 | | |
| Farmyard manure | 590 | 830 | | |
| Rape cake | — | 755 | | |

others, the rate of loss is then based upon the difference between the earliest and latest samples from each plot.

### B. *Loss of Calcium Carbonate on Unmanured Land.*

It will be convenient to consider the unmanured plots together, so as to get an idea of the initial loss of calcium carbonate to the soil when there are no disturbing influences introduced by the manure. All the unmanured soils show the same general characteristics—a comparatively large amount of calcium carbonate in the top 9 inches, varying from nearly 6 per cent. on Agdell Field down to little more than 2 per cent. on Hoos Field. The second depth, however, contains as a rule between 0·1 and 0·2 per cent., only in a few special cases does it rise above the latter figure; in the third depth the proportion is 0·1 per cent. or less. As the Rothamsted fields are situated on almost the highest levels of the chalk plateau, it is difficult to suggest any reason for the restriction of the calcium carbonate to the surface soil other than its artificial origin, and this conclusion is confirmed both by the irregularity in the amount found in different fields and by its absence where the land has not been under cultivation. Samples were taken from three places on the adjoining Harpenden Common, where the same class of land occurs at approximately the same level as the experimental fields, but which has never been in cultivation and now carries a growth of poor grass, gorse, and bracken.

The results:—

| | | | |
|---|---|---|---|
| Soil of Harpenden Common, | 1 to 9 inches deep... | 0·210 p. c. $CaCO_3$. |
| „ „ | 10 to 18 „ ... | 0·136 „ |

show that initially the soil of the Rothamsted Estate must have been practically devoid of calcium carbonate.

The successive determinations of the amounts present in the soil of the cultivated fields give the following rates of loss per acre per annum for the unmanured land: Broadbalk 800 lbs.; Hoos, 1000 lbs.; Agdell, 922 and 938 lbs.; Little Hoos, 1046 lbs., or an average loss of over 944 lbs. per acre per annum due to percolating water only. These rates are all probably too high, because of the gradual exhaustion of the organic matter and the resultant consolidation of the soil which has affected the later samples. But even after making all corrections that are possible for this source of error, the rate of loss would still amount to 800 lbs. per acre.

These estimates may be to some extent checked by certain determinations made in 1896—8 of the amount of calcium compounds present in the water percolating through the drain gauges at Rothamsted. These drain gauges are blocks of undisturbed soil of 1/1000 acre in area, isolated by impervious walls from the surrounding land and maintained without vegetation since 1870. Table IX shows the proportion of lime in the water during 20 months

Table IX.—Lime in Drainage Water percolating through 60 inches of Soil, September, 1896, to April, 1898.

| | Rainfall. | Drainage through soil, 60 inches deep. | CaO in drainage water per million. |
|---|---|---|---|
| | inches. | inches. | |
| 1896. | | | |
| September | 8·077 | 6·362 | 60·1 |
| October | 4·132 | 2·992 | 56·3 |
| November | 1·387 | 0·833 | 46·1 |
| December | 4·416 | 3·811 | 49·8 |
| 1897. | | | |
| January | 2·031 | 1·585 | 48·9 |
| February | 2·925 | 3·264 | 47·9 |
| March | 4·197 | 2·589 | 48·2 |
| April | 1·913 | 0·320 | 48·4 |
| May | 1·718 | 0·047 | 53·3 |
| June | 2·734 | 0·865 | 54·1 |
| July | 0·467 | 0·024 | 44·5 |
| August | 3·238 | 0·105 | 61·0 |
| September | 2·440 | 0·872 | 60·5 |
| October | 0·960 | 0·001 | — |
| November | 1·048 | 0·110 | 50·4 |
| December | 3·503 | 3·060 | 56·6 |
| 1898. | | | |
| January | 0·795 | 0·821 | 53·1 |
| February | 1·098 | 0·047 | 30·5 |
| March | 1·060 | 0·492 | 51·5 |
| April | 1·443 | 0·082 | 43·2 |
| Average | 2·479 | 1·414 | 53·5 |

(September, 1896, to April, 1898); the average concentration is 53·5 parts of CaO per million for the gauge with 60 inches of soil, which on the average percolation of 13·8 inches would give an annual loss of calcium carbonate of 300 lbs. Two causes contribute to make this figure low. In the first place the soil of the gauges is not very rich in calcium carbonate, determinations made on samples taken in 1870 from the land immediately adjoining gave 3·06 per cent., while two small samples bored out from the actual gauges in 1905 gave 1·88 per cent. for the upper 9 inches. Secondly, the air contained in the soil of this plot must be comparatively deficient in the carbon dioxide necessary to bring the chalk into solution; as a rule the soil gases get richer in carbon dioxide the greater the depth, but the soil of the gauge is cut off from the subsoil and open to the atmosphere at the 60-inch depth. The long absence of any crop or manure will have reduced to very small limits the amount both of organic matter decaying to carbon dioxide, and the organic sulphur compounds which by bacterial action become sulphuric acid and leave the soil as calcium sulphate. These causes will co-operate to lessen the removal of calcium carbonate from the soil in the gauge, and as a matter of fact the concentration of 53·5 parts of CaO per million observed in its drainage is only about half of the concentration of the water running from the tile-drains beneath the unmanured plot in Broadbalk, which according to Voelcker's and Frankland's analyses (Table X) amounted in the mean to about 99 parts per million. But assuming that this latter figure represents the average proportion of lime in the drainage water from the unmanured plot, and that the average annual percolation through the soil of this plot is 10 inches,* equal to that through the 60-inch gauge, the annual loss of calcium carbonate per acre should amount to 400 lbs. for the unmanured plot instead of the 800 lbs. found by analysis of the soil of the plot. The number of analyses, however, upon which the former estimate is based, is too small for great accuracy.

Table X.—Broadbalk Drainage Water.
Mean of 10 analyses by Voelcker and Frankland. Parts per million.

| Plot. | Manures. | Total solid matter. | Lime and magnesia. |
|---|---|---|---|
| 2 | Farmyard manure (14 tons) ............... | 367·2 | 123 |
| 3 and 4 | Unmanured ...................................... | 227·8 | 99 |
| 5 | Minerals only ................................ | 329·8 | 132 |
| 6 | „ + 200 lbs. ammonium salts ... | 450·3 | 171 |
| 7 | „ + 400 lbs. „ „ ... | 542·4 | 207 |
| 8 | „ + 600 lbs. „ „ ... | 615·1 | 222 |
| 9 | „ + 275 lbs. nitrate of soda ...... | 405·7 | 126 |
| 10 | 400 lbs. ammonium salts alone ............ | 441·8 | 173 |
| 11 | „ „ + superphosphate ... | 490·4 | 197 |

In a paper by Creydt, von Seelhorst, and Wilms† on the composition of the drainage waters from an ordinary field tile-drained at a depth of

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* Ten inches was the estimate formed by Lawes, Gilbert and Warington, 'Journ. Roy. Agri. Soc.,' 1882, vol. 43, p. 24; Warington, 'Trans. Highland and Agri. Soc.,' 1905, vol. 17, 5th series, p. 168, estimates the drainage as somewhat more than 8·2 inches, while a comparison between the concentration of chlorine in the water from the unmanured plot and from the 60-inch drain gauge would lead to an estimated drainage of 9 inches.

† 'Journ. der Landw.,' 1901, p. 251.

53 inches, the annual loss of lime is estimated at 630 kilogrammes per hectare, almost exactly equivalent to 1000 lbs. of calcium carbonate per acre. Unfortunately, the proportion of calcium carbonate in the soil is not given, but the agreement with our figures for the removal of calcium carbonate is very satisfactory.

Another consequence of some interest follows from these determinations of the loss of calcium carbonate from the unmanured plots. The analyses already quoted of the drainage water from the unmanured plot of Broadbalk Field show about 100 parts of lime per million, equivalent to a loss of about 400 lbs. of calcium carbonate per acre in the surface soil instead of 800 lbs. estimated from the analysis of the soil. But the deep-seated waters of the chalk contain on the average about 150 parts of lime per million, a deep well at Harpenden, for example, yielding 158 parts per million.* Assuming that the percolation through the unmanured plot on Broadbalk represents the average percolation over the chalk area, then the removal of chalk by solution would be in the ratio of the concentrations of the two waters in question, *i.e.*, the average annual denudation of the chalk by solution alone would amount on the one estimate to 600 lbs., or on the other to 1200 lbs. per acre per annum. As the specific gravity of the chalk is about 2·2, and it contains from 95 to 99 per cent. of calcium carbonate, this would mean a lowering of the surface by solution alone at the rate of either 1/11000 or 1/5500 of a foot per annum. These estimates depend upon the assumption that the percolation through this unmanured plot of arable land represents the percolation over the whole area of the chalk, whereas ordinary crops or even grass would cause increased transpiration and allow of less percolation. But on the contrary, the figure adopted for the concentration of the lime in the drainage water from the unmanured plot is based on only a small number of analyses and would appear to be too low. The concentration of the drainage waters would be increased by any use of organic manures, by heavy cropping or by permanent vegetation, all of which would increase the production of the carbon dioxide causing dissolution in the soil. For example the analyses of the water from the plot receiving farmyard manure every year show an average of 123 parts of lime per million instead of 99 from the unmanured plot. This increased proportion of lime in the water percolating through ordinary land may be set off to some extent against the lessened percolation due to crops, but on the whole the evidence is in favour of the lower rate, so that a denudation of about 1/10000 foot per annum is a more probable figure.

The accuracy of the sampling is not sufficient to enable any conclusion to be drawn as to whether the loss of calcium carbonate fluctuates with the rainfall and percolation during each period. But the magnitude of the annual loss is somewhat surprising; assuming it to be only 800 lbs. per acre, then the Broadbalk Field must have contained at least 70 tons per acre of chalk at the beginning of the nineteenth century, and still contains so much that it will not be exhausted by the end of the present century. As, also, the rate of loss will probably fall with each reduction in the quantity present, dissolution being proportional to the surface exposed, the period of complete exhaustion will be considerably postponed. Since much of the value of the land agriculturally, both in its fertility and in its ease of working, depends on the presence of calcium carbonate, it is clear that for the last 100 years the agricultural community have in this respect been living upon the capital accumulated by their forefathers, and are taking no steps to replace the inevitable depletion of this capital.

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* Warington, 'Trans. Chem. Soc.,' vol. 51, 1887, p. 543.

### C. *Effect of Manures upon the loss of Calcium Carbonate.*

Most of the manured plots under investigation receive a dressing of "mineral manures" in addition to the varying amounts and compounds of nitrogen. This mineral manure consists of 3½ cwt. per acre of superphosphate containing 17 per cent. of soluble phosphoric acid (equivalent to 37 per cent. of calcium phosphate "made soluble"), 200 lbs. of potassium sulphate, and 100 lbs. each of magnesium and sodium sulphates. Of these substances the superphosphate reacts immediately with the calcium carbonate of the soil, the sparingly-soluble di-calcium hydrogen phosphate being precipitated wherever the superphosphate solution comes in contact with a particle of chalk in the soil in accordance with the equation

$$CaH_4P_2O_8 + CaCO_3 = Ca_2H_2P_2O_8 + CO_2 + H_2O.$$

To complete this reaction, the 3½ cwt. of superphosphate would require about 47 lbs. of calcium carbonate, but so small an annual loss would hardly be perceptible in the analyses.

The neutral sulphates of potassium, sodium, and magnesium should occasion no loss, for though they react with calcium carbonate, the resultant alkaline carbonate is retained by the soil and would be estimated as calcium carbonate by the method of analysis adopted, which is based upon the carbon-dioxide evolved on treating the soil with acid. The action of the plant also, discussed later in this paper, would probably result in the reconversion of the sodium and potassium carbonates into calcium carbonate. The action of the mineral manures, as seen in the analyses of the soil, has not occasioned sufficient loss of calcium carbonate to be apparent within the limits of accuracy of the determinations. In the Broadbalk Field, Plot 10 can be compared with Plot 7; both receive the same amount of ammonium salts, but Plot 7 receives the minerals in addition. The rate of loss is practically identical, 1010 lbs. per acre per annum on Plot 7 and 1045 lbs. on Plot 10. Unfortunately Plot 5, receiving minerals only without nitrogen, cannot be compared with the unmanured Plot 3, because both Plots 5 and 6 show an entirely exceptional rate of loss, not to be accounted for unless it be that one of the dells, from which the field was originally chalked, lies in these two plots, and may have caused a very irregular distribution of the chalk. But if the rate of loss on Plot 5 be calculated over the period 1881—1904 only, it amounts to 880 lbs. per acre against 800 lbs. per acre on the unmanured plot.

In the Hoos Field the results are too irregular to bear much discussion, for the plots which receive minerals—4o, 4A, 4N—start with less than half the chalk contained by the corresponding 1o, 1A, and 1N.

In the plots, however, which receive ammonium salts as a manure, the loss of calcium carbonate is much increased. A reaction of the type

$$(NH_4)_2SO_4 + CaCO_3 = (NH_4)_2CO_3 + CaSO_4$$

takes place as soon as the ammonium salts are dissolved, the ammonium carbonate is adsorbed by the surface action of the humus and the finer clay particles of the soil until it is nitrified, while the calcium sulphate passes forthwith into the drainage water. When heavy rain follows the application of the ammonium salts to the Broadbalk wheatfield, only traces of ammonia find their way into the drains, whereas there is an immediate great increase in the calcium sulphate and chloride present in the drainage water. At Rothamsted the manure termed ammonium salts consists of an equal mixture of ammonium sulphate and chloride, and is applied to the various plots at the rate of 200, 400, and 600 lbs. per acre, quantities which would react with 161, 321, and 482 lbs. respectively of calcium carbonate. Before, however, the ammonium carbonate thus produced has been long in the soil a second molecule of calcium carbonate must be consumed to provide a base for the nitrous and nitric acids formed by its nitrification. This would make the loss caused by the application of 200 lbs. of ammonium salts up to 321 lbs. of calcium carbonate, half of which is caused by the initial reaction producing ammonium carbonate, and half by the nitrification of the latter. In order to determine the rate of loss in the field, it will be convenient, in the various cases where a comparison is possible, to subtract the average rate of loss on a plot without ammonium salts from the plots receiving ammonium salts, and divide by 2 or 3, as need be, to find the effect in each case of 200 lbs. of ammonium salts.

| | | Annual loss of calcium carbonate. | |
|---|---|---|---|
| Broadbalk | (Plot 7 − Plot 3) ÷ 2 | 105 | lbs. per acre. |
| „ | (Plot 10 − Plot 3) ÷ 2 | 122 | „ |
| „ | (Plot 8 − Plot 3) ÷ 3 | 125 | „ |
| Hoos | (Plot 4A − Plot 4O) ÷ 1 | 115 | „ |
| | Mean | 117 | „ |

This mean value for the loss caused by 200 lbs. of ammonium salts is not very far from the 161 lbs. estimated above as necessary to convert them into ammonium carbonate, but neither the mean nor any of the individual analyses support the view that a second molecule of calcium carbonate is removed from the land by the nitrification of the ammonium carbonate. However, this nitrification must take place; indeed, there is every evidence that it takes place so rapidly and thoroughly in the Rothamsted soil that no ammonium salts are carried forward un-nitrified from one season to the next. The analyses of the drainage waters (Table X), while they show a progressive increase in the amount of lime for each addition of ammonium salts in the manure, do not permit any estimate to be formed of the rate of removal, so much is the extent of the percolation, as seen in the relative frequency with which the drains run, affected by the size of the crop, which becomes large on the heavily-manured plots. It remains, therefore, to be explained why the loss of the soil should be at the rate of one rather than of two molecules of calcium carbonate for every two molecules of combined ammonia applied in the manure.

The plots receiving sodium nitrate in place of ammonium salts show not only no special loss of calcium carbonate due to the nitrogeneous manure, but a distinctly diminished loss as compared with the unmanured plot. On Broadbalk the nitrate plot loses at the rate of 564 lbs. per acre against 800 lbs. on the unmanured plot; on Hoos the loss is 465 lbs., against 675 lbs. on the plot receiving the same minerals but no nitrate.

That the sodium nitrate exercises some conserving influence on the calcium carbonate in the soil is also apparent from a consideration of the analyses of the subsoil. On Broadbalk, where the subsoil from 10 to 18 inches contains in most cases 0·1 or 0·15 per cent. of calcium carbonate, the same layer beneath the plot receiving sodium nitrate shows 0·31, 0·26, 0·48, and 0·14 per cent. at the various dates, while the third depth of this plot is also richer than on the corresponding plots.

Further evidence may be derived from the composition of the drainage waters (Table X); the water from the plot receiving mineral manures only contains 132 parts of lime per million, the plot receiving the same minerals and sodium nitrate gives a drainage water containing 126 parts of lime per million. Thus where the nitrate is used there is both a lower concentration of lime in the drainage water and a smaller total percolation, because of the much greater crop, and consequently increased transpiration on this plot.

The sodium nitrate then either saves the calcium carbonate of the soil from its normal loss or has some power to bring about the re-formation of calcium carbonate. With this fact must be correlated the non-disappearance from the other plots of the calcium carbonate required to form calcium nitrate with the nitrified ammonia base.

Considering lastly the plots receiving farmyard manure, the Broadbalk Field shows a much lower rate of loss on the plot manured every year in this way than on the unmanured plot, 590 lbs. against 800 lbs. per acre per annum. The corresponding plots in the Hoos Field hardly confirm this view, since both the plot receiving farmyard manure and that receiving rape cake, the only other organic manure employed, appear to be losing calcium carbonate at much the same rate as all the other plots. However, as the Hoos Field results rest upon determinations made at two dates only instead of four as in the case of Broadbalk, it is much more probable that the result yielded by the latter is trustworthy. The subsoil of the plots receiving farmyard manure also show amounts of calcium carbonate above the normal—0·28, 0·31, 0·42, and 0·24 in Broadbalk, and 0·28 and 0·15 in Hoos Field. The drain beneath the farmyard manure plot on the Broadbalk Field runs but rarely, because the humus derived from the long-continued organic manuring of this plot is capable of temporarily absorbing any ordinary rainfall and then passing it gradually down to the subsoil without causing the drain to run. But the few analyses that have been made of the water draining from this plot indicate a lower concentration in calcium compounds than would be expected from the large amount of carbon dioxide produced by the decay of recent organic matter, and also from the considerable annual addition of calcium compounds in the manure itself. The composition of farmyard manure is very variable, but the mean of a number of analyses gives 0·6 per cent. of CaO, or an annual application of 190 lbs. per acre, of which the greater part is in combination with organic acids. There is, therefore, an addition of calcium compounds in the manure more than equivalent to their greater concentration in the drainage water, and the net result is a gain of calcium carbonate to the soil as shown by the diminished rate of loss on this as compared with the unmanured plot.

We thus obtain three lines of evidence that there is some agency saving or re-creating the calcium carbonate of the soil: (1) the loss of calcium carbonate induced by the use of ammonium salts is less than half that required for the absorption and subsequent nitrification of the ammonia; (2) where sodium nitrate or (3) where farmyard manure is applied, the rate of loss of calcium carbonate is below that of unmanured land.

Further evidence that there must be under normal conditions some action at work protecting or renewing the bases of the soil may be gathered from the continued fertility of many soils containing but a trace of calcium carbonate. The following analyses may be quoted of soils that have fallen under the personal observation of one of us, soils which, despite their very low content of calcium carbonate, have continued to give crops under arable cultivation for a long period.

Table XI.—Calcium Carbonate per cent. in various Soils.

| Formation. | London clay. | London clay. | Gault clay. | Weald clay. | Bagshot sand. | Thanet beds. | Woburn Experimental Farm, Stackyard Field. Barley. | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Locality. | Wanboro', Surrey. | Ashtead Common. | Alder Holt. | Staplehurst, Kent. | Bisley. | Woodnesborough. | 1876. | 1903. Plot 2*a*. | 1903. Plot 3. | 1903. Rotation. |
| | p. c. | p. c. | p. c. | p. c. | p. c. | p. c. | p. c. | p. c. | p. c. | p. c. |
| 1st depth ... | 0·065 | 0·002 | 0·04 | 0·037 | 0·008 | 0·018 | 0·087 | 0·051 | 0·070 | 0·089 |
| 2nd depth ... | 0·084 | nil | 0·16 | 0·012 | 0·016 | 0·010 | 0·066 | 0·044 | 0·042 | 0·071 |

Another striking case is afforded by the Stackyard Field on the farm of the Royal Agricultural Society at Woburn, which has been under experiment since 1876. Table XI also shows a series of determinations of calcium carbonate in the soil of this field taken at the beginning of the experiments and in 1902. The amount of calcium carbonate present is exceedingly small, barely determinable in fact, yet the plots continue to yield normal crops, except those which have been manured with ammonium salts. The latter in recent years have become almost sterile, showing an acid reaction to litmus paper and refusing to grow wheat or barley unless they first receive a dressing of lime.*

Now in all these cases, however low the proportion of calcium carbonate may be, the action of the percolating water must remove some of it, and the recurring process of nitrification also demands a base. Yet the small quantity of base available does not disappear entirely so as to render the soil unfertile, unless some specially calcium carbonate consuming material, like the ammonium salts, is employed as a manure. The continued fertility of such soils almost devoid of calcium carbonate has long been a problem, but it now seems probable that the calcium carbonate and other bases which are required for nitrification are in some way returned to the soil as bases, and that when a ready-formed nitrate like sodium nitrate is used as a manure there is an addition of available base to the soil or a corresponding diminution in the amount of calcium carbonate removed by the drainage water. Furthermore these or other agencies conservative of calcium carbonate are sufficient to maintain the quantity in the soil at the level for comparatively healthy growth. Of the possible conservative actions, two will be now considered and evidence be brought to show that (1) the normal growth of plants leaves behind a residue of base in the soil, (2) the decay of plant tissues results in the production of calcium carbonate.

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* See J. A. Voelcker, 'Journ. Roy. Agri. Soc.,' 3rd Series, vol. 10, 1899, p. 585, and vol. 62, 1901, p. 272.

## II.—Effect of Plant Growth on the Reaction of the Soil.

The plant, it is well known, does not take up the salts of the soil water in the proportions in which they are present in the solution, but exercises a selective action in favour of substances necessary to the nutrition processes, such as potash and phosphoric acid. And if the composition of the ashes of the plant be taken into account, it is clear that the selective action is exercised not merely on the salts with which the root is in contact but on their acids and bases considered separately. For example, from a solution of calcium nitrate the plant would withdraw more nitric acid than its equivalent of lime and from a solution of potassium sulphate more potash than its equivalent of sulphuric acid.

When a plant is burnt the ash is usually alkaline, because the organic acids and any nitrogen present as nitrate in the plant are all driven off, leaving the bases as carbonates. But when a balance is struck between the acids and bases in the ash and when the nitrogen present in the plant before burning is calculated as an acid, since it all entered the plant as nitrate, the acids are generally to be found in excess. Warington,* indeed, has already pointed out with reference to the published analyses, that plants must retain more acids than bases. It does not appear to have been noticed, however, that such a result, by leaving behind in the soil a corresponding excess of base, must have an appreciable effect upon the reaction of the soil, although Knop and other early investigators have observed that the solutions in which plants are grown as water cultures become alkaline after a time. It is hardly possible to decide whether the excess of base is left behind in the soil water or excreted by the root cells. The nutrient constituents pass through the cell wall of the root hairs by osmosis until the concentration is the same on either side; such substances as are required by the plant are then withdrawn from action by the protoplasm, thus lowering the concentration on the inside and causing a fresh diffusion of that particular substance through the cell wall. Looking at the question from the standpoint of the ionic hypothesis, the soil water would be a highly ionised solution, in which any given ion will pass by osmosis into the root-hairs as long as the plant maintains the tension of that ion lower on the inside of the cell than in the solution outside. As the plant is always transforming the nitrogen, sulphur and phosphorous from the condition of inorganic acids in which they enter into neutral or even basic organic compounds, and since it also as a rule utilises more of these substances than of the metallic bases, such acid ions will be withdrawn by the protoplasm in greater quantity, and so must pass through the cell wall from the external solution at a greater rate than the corresponding basic ions, the necessary equilibrium being maintained by the carbonic acid excreted by the root cells.* From this point of view, when the root is drawing nutriment from a solution of a neutral nitrate, the nitric acid ions would be travelling inwards to the protoplasm and the carbonic acid ions outwards, so that the carbonate of the base might be considered as forming outside the cell wall. If, on the other hand, the salt be considered to move into the cell undissociated and there to lose its nitric acid to the protoplasm, the base must then be supposed to diffuse out again as carbonate. The net result, however, is the same under either hypothesis, viz., that after the plant has been growing for some time in a neutral solution it will have taken up an excess of acid and left a corresponding excess of base, now combined as carbonate, in the solution representing the water of the soil.

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* 'Agricultural Students' Gazette,' 1899, p. 133 ; see Lawes and Gilbert, 'Journ. Roy. Agri. Soc.,' vol. 55, 1894, p. 640.

The following Table (XII) shows the composition of several crops as percentages and again recalculated as equivalents of hydrogen, phosphoric acid being reckoned as tribasic, since the soil solution will be mainly derived from tribasic phosphates in the soil.

It will be seen that there is a considerable excess of acid in the plant, from which it follows that an equivalent amount of base was left behind in the soil. This base is in most cases nearly equivalent to the nitrogen taken in as nitrate, and calculated as calcium carbonate will amount to between 100 and 300 lbs. of calcium carbonate per acre. In other words the normal growth of farm crops leaves behind from the salts in the soil used for its nutrition about as much base as would have been previously required for the nitrification of

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* For a discussion of the electrical disturbances such an interchange would involve, see Kohn, 'Landw. Versuchs.,' vol. 52, 1899, p. 315.

Table XII.—Basic and Acid Constituents of various Crops.

| | Agdell rotation. Wheat. Complete manure. 8 courses. | Agdell rotation. Barley. Complete manure. 8 courses. | Agdell rotation. Swedes. Complete manure. 3 courses. | Park hay, Plot 9, 1856–1873. |
|---|---|---|---|---|
| Dry matter per acre | 4749 | 4124 | 3899 | 4812 |
| Nitrogen per cent. in dry matter | 0·88 | 1·00 | 2·41 | 1·55 |
| Ash per cent. in dry matter | 4·74 | 4·00 | 6·59 | 7·24 |
| Percentage Composition of Ash. | | | | |
| Ferric oxide | 0·37 | 0·64 | 0·96 | 1·32 |
| Lime | 4·41 | 7·82 | 13·42 | 8·27 |
| Magnesia | 2·98 | 4·16 | 2·70 | 3·42 |
| Potash | 16·21 | 19·33 | 36·33 | 35·59 |
| Soda | 0·39 | 1·67 | 4·27 | 3·87 |
| Phosphoric acid | 9·09 | 12·88 | 9·38 | 7·96 |
| Sulphuric acid | 2·56 | 3·58 | 12·36 | 5·76 |
| Chlorine | 1·46 | 3·15 | 4·18 | 14·83 |
| Silica | 60·58 | 46·47 | 1·04 | 16·54 |
| Constituents reduced to Equivalents of Hydrogen, and Lbs. per Acre. | | | | |
| Ferric oxide | 0·03 | 0·04 | 0·09 | 0·17 |
| Lime | 0·36 | 0·46 | 1·24 | 1·03 |
| Magnesia | 0·33 | 0·34 | 0·35 | 0·60 |
| Potash | 0·78 | 0·68 | 1·99 | 2·64 |
| Soda | 0·03 | 0·09 | 0·36 | 0·44 |
| Bases, total | 1·53 | 1·61 | 4·03 | 4·88 |
| Phosphoric acid | 0·87 | 0·90 | 1·02 | 1·18 |
| Sulphuric acid | 0·14 | 0·15 | 0·80 | 0·50 |
| Chlorine | 0·09 | 0·15 | 0·30 | 1·47 |
| Nitrogen | 3·00 | 2·97 | 6·74 | 5·35 |
| Silica | 4·53 | 2·55 | 0·09 | 1·91 |
| Acids, total, excluding silica | 4·10 | 4·17 | 8·86 | 8·50 |
| Total of bases | 1·53 | 1·61 | 4·03 | 4·88 |
| Excess of acid (as hydrogen) | 2·57 | 2·56 | 4·83 | 3·62 |
| Equivalent to calcium carbonate | 129 | 128 | 242 | 181 |

the nitrates which entered the plant, as measured by the nitrogen finally contained in it.

Although the many ash-analyses which have been made of farm crops afford conclusive evidence of this restoration of base to the soil, it seemed desirable to submit the fact to experimental verification. In 1903 water cultures were made of barley and cabbage in normal solutions which were contained in cylinders of Jena glass holding about 3 litres; it was found that after growth had gone on for some time the solutions had gained in alkalinity by an amount equivalent to the nitrogen taken up by the plant, less the alkalinity of the plant ash. One example of these trials will suffice.

A cabbage plant made 7·525 grammes dry matter containing 2·705 per cent. of nitrogen = 0·2035 gramme nitrogen, equivalent to 0·01454 gramme hydrogen. The ash of the plant had an alkalinity equivalent to 0·01082 gramme hydrogen. The culture solution gained alkalinity = 0·00558 gramme hydrogen, which with the 0·01082 gramme alkalinity of the ash makes 0·0164 gramme of alkaline hydrogen found in ash and solution, to correspond with the 0·01454 gramme of acid hydrogen equivalent to the nitrogen in the plant.

In 1904 six cultures were started with wheat in similar jars on March 3, and growth was continued without changing the solutions until June 11, when the grain was fully formed. Three grains were sown in each jar and growth was extremely vigorous, but was continued a little too long, for at the close of the experiment no nitrate remained in the solution. For purposes of analysis a mixture was made of Nos. 1 and 2, and of 3 and 4, the remaining two being kept in reserve. Table XIII shows the results obtained.

In the initial solution there was a trifling excess of acid over base owing to the presence of a little ferric chloride, of which the acid alone is brought into the account. At the end it will be seen that there is an apparent gain of nitrogen, due in the main to dust (the greater part being present in the sediment which formed in each jar) and the seed (which contained

Table XIII.—Wheat Grown in Water Culture. Composition of Plant and Solution after Growth.

Original Solution.

| | Quantities taken. | Equivalent to hydrogen. |
|---|---|---|
| | grammes. | gramme. |
| $K_2O$ | 4·2190 | 0·0898 |
| MgO | 0·6018 | 0·0301 |
| CaO | 2·8000 | 0·1000 |
| Total bases | — | 0·2199 |
| N (as nitrate) | 1·4000 | 0·1000 |
| $P_2O_5$ (monobasic) | 2·0974 | 0·0295 |
| $SO_3$ | 1·2080 | 0·0302 |
| Cl | 2·1658 | 0·0608 |
| Total acid | — | 0·2205 |

TABLE XIII—*continued.*

After Growth. Nos. 1 and 2. Dry Matter produced = 93·7 Grammes.

| | Quantities found. | | | Equivalent to hydrogen. | | | |
|---|---|---|---|---|---|---|---|
| | Sediment. | Solution. | Plant. | Sediment. | Solution. | Plant. | Total. |
| | gramme. | grammes. | grammes. | gramme. | gramme. | gramme. | gramme. |
| $K_2O$ | — | 0·5014 | 3·6644 | — | 0·01065 | 0·07796 | 0·08861 |
| MgO | (0·2082*) | 0·2318 | 0·1618 | (0·01041) | 0·01159 | 0·00809 | 0·03009 |
| CaO | 0·152 | 1·1592 | 1·4371 | 0·00543 | 0·04140 | 0·05140 | 0·09823 |
| | | | | 0·01584 | 0·06364 | 0·13745 | 0·21693 |
| N | 0·1249 | 0·0103 | 1·3540 | 0·00892 | 0·00074 | 0·09670 | 0·10636 |
| $P_2O_5$ | 0·2942 | 0·5631 | 1·246 | 0·00414 | 0·00793 | 0·01755 | 0·02962 |
| $SO_3$ | — | 0·6448 | 0·4008 | — | 0·01612 | 0·01002 | 0·02614 |
| Cl | — | 0·8114 | 1·3461 | — | 0·02285 | 0·03792 | 0·06077 |
| | | | | 0·01306 | 0·04764 | 0·16219 | 0·22289 |
| | Bases in excess | | | +0·00278 | +0·01600 | −0·02474 | −0·00596 |
| After Growth. Nos. 3 and 4. Dry Matter produced = 77·6 Grammes. | | | | | | | |
| $K_2O$ | — | 0·6736 | 3·4819 | — | 0·01433 | 0·07410 | 0·08843 |
| MgO | (0·1296*) | 0·2413 | 0·2309 | (0·00648) | 0·01206 | 0·01154 | 0·03008 |
| CaO | 0·2240 | 1·2222 | 1·3414 | 0·00800 | 0·04365 | 0·04790 | 0·09955 |
| | | | | 0·01448 | 0·07004 | 0·13354 | 0·21806 |
| N | 0·0972 | 0·0080 | 1·4038 | 0·00694 | 0·00055 | 0·10027 | 0·10776 |
| $P_2O_5$ | 0·345 | 0·5765 | 1·170 | 0·00486 | 0·00812 | 0·01648 | 0·02946 |
| $SO_3$ | — | 0·6610 | 0·3959 | — | 0·01652 | 0·00990 | 0·02642 |
| Cl | — | 0·8595 | 1·2212 | — | 0·02421 | 0·03440 | 0·05861 |
| | | | | 0·01180 | 0·04940 | 0·16105 | 0·22225 |
| | Bases in excess | | | +0·00268 | +0·02064 | −0·02751 | −0·00419 |

* Estimated figure—these two determinations were lost.

0·0036 gramme nitrogen, equivalent to 0·00026 gramme hydrogen), and partly to a trace of ammonia in the large quantities of distilled water evaporated during growth. This excess of nitrogen accounts for the slight excess of acid over base in the final result when all the nitrogen is reckoned as acid. It will be seen that the solution and sediment (representing the soil) gained in the one case base equivalent to 0·01878 gramme of hydrogen and in the other to 0·02332 gramme of hydrogen, quantities which would probably have been increased had the growth not been continued until all the nitrate was exhausted.

It has been often supposed that plants excrete some organic acid from the root which aids in bringing about the solution of nutrient materials in the soil,* but no evidence could be found that anything except carbon dioxide passed from the roots into the culture solution. After growth a considerable bulk of the culture solution was evaporated to dryness and gently heated, very slight signs of charring were observed, no more than could be attributed to the dust, and the residue showed the same alkalinity before and after ignition, as would not be the case had any organic acid excreted from the root been present. On one occasion freshly precipitated ferric hydrate was introduced into the solution as a source of iron; although this was distributed all over the young growing roots so that it could not be shaken off, the plants began to suffer from lack of iron, and continued to do so until a trace of ferric chloride was introduced. Despite the well known acidity of the root-sap there seems no evidence that in normal cases it ever passes outside the cell wall, as long as the roots are unbroken.

These experiments then afford experimental justification for looking to the growth of the plant as an explanation of some of the difficulties raised by the rate of loss of calcium carbonate on the different plots. The analyses of crops already quoted serve to show that the return of base to the soil may be large, quite sufficient to make up for the calcium carbonate required each year for nitrification. Hence soils which start with very small proportions of calcium carbonate may yet preserve their healthy condition and permit of nitrification, the losses caused by which are thus repaired.

Again it becomes intelligible that the use of ammonium salts as a manure only occasions the loss of one molecule of calcium carbonate for each two molecules of ammonia, since the second molecule required for nitrification will be more or less restored during the growth of the plant. It has already been shown that the actual loss of calcium carbonate to the soil caused by the use of 200 lbs. of ammonium salts approximates to 161 lbs., and not the 322 lbs. which would be required if the calcium nitrate produced by nitrification were wholly removed from the soil.

Further, when nitrate of soda is used as a manure, from the neutral solution in the soil of calcium or sodium nitrate an excess of acid will be taken by the crop, leaving the soil richer in base. Hence the conservative action of sodium nitrate on the calcium carbonate of the soil that is visible in the analyses of both Broadbalk and Hoos Fields. It is possible to calculate the amount of base restored to the plots receiving nitrate of soda on the assumption that they possess the same average composition as the wheat and barley in Table XII, and that the amount of base returned will be in proportion to the size of the crop. In this way the following results are obtained:—

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* See Czapek, 'Pringsheim's Jahr. f. wiss. Botanik,' vol. 29, 1896, p. 321; Kossowitsch, 'Ann. de la Science Agronomique,' 2nd Series, vol. 1, 1903, p. 220.

Table XIV.

| | Broadbalk Field. | | Hoos Field. | |
|---|---|---|---|---|
| | Plot 3. | Plot 9. | Plot 4o. | Plot 4n. |
| Total produce (grain and straw) | lbs.<br>1936 | lbs.<br>6133 | lbs.<br>2343 | lbs.<br>5524 |
| Bases restored to the soil as calcium carbonate, calculated from total produce | 53 | 167 | 73 | 171 |
| Mean rate of loss of calcium carbonate from the soil | 800 | 564 | 675 | 465 |
| Total annual consumption of calcium carbonate | 853 | 731 | 748 | 636 |

The agreement between the figures in the last line is not very close, but indicates that the restoration of base to the soil, as calculated from the increase of crop on the plots receiving nitrate of soda, is approximately equivalent to the lower rate of loss of calcium carbonate found on analysis of the soil of these plots.

The results, as a whole, go to show that the action of plants, in leaving behind a basic residue from the neutral salts in the soil upon which they feed, is a very essential feature in the chemistry of the soil, explaining, amongst other things, the maintenance of healthy conditions on the many soils poor in calcium carbonate. It also serves to explain one or two other points which have been observed in connection with the use of sodium nitrate as a manure. It has long been noticed that the continued use of sodium nitrate is very destructive to the texture of a clay soil, intensifying all the clay properties, rendering the soil persistently unworkable when wet, and forming hard and intractable clods when dry. The ultimate cause of such an effect is the "deflocculation" of the fine particles composing the soil; they are no longer bound together in loose aggregates, but are separated so as to give the soil its most finely grained character. Such deflocculation of the soil can be brought about by a trace of any soluble alkali, just as the opposite state of flocculation is induced by a slightly acid reaction. The Rothamsted soils continuously manured with sodium nitrate show marked signs of deflocculation, the drainage water from the nitrated plots in the Broadbalk Field is always more turbid than that from the other plots, and as one of us has shown,* there results in time on the nitrated plots a perceptible washing down of the finest particles set free by the deflocculation into the subsoil or the drains. The bad texture of the soil following on the use of sodium nitrate is particularly to be seen on the mangel field, where it reaches its maximum on the plots receiving sodium nitrate and other neutral alkali salts like potassium sulphate and sodium chloride: it has been repeatedly observed to be at its worst in the winter and spring after a large crop has been grown on the sodium nitrate plots. As the soil of this field contains but little calcium carbonate, some of the base left behind in the soil by the growth of the crop would consist of bicarbonate of sodium or potassium, especially where the other alkali salts are applied in the manure, and there would be quite enough free alkaline carbonate thus formed to cause a thorough deflocculation of the soil. This explanation would agree with the observed fact that the deflocculation is much diminished where superphosphate only, an acid manure, is used in conjunction with the sodium nitrate.

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* Hall, 'Trans. Chem. Soc.,' 1904, vol. 85, p. 964.

## III.—EFFECT OF ORGANIC MANURES ON THE REACTION OF THE SOIL.

Although the evidence is not so trustworthy as in the case of sodium nitrate, yet the use of farmyard manure and of rape cake seems also to result in a diminished rate of loss of calcium carbonate to the soil. Some of this may be due to the lessened percolation consequent on the greater water-retaining power of the soil enriched in humus, but another cause may be sought in the bacterial decomposition of calcium salts in the organic *débris.* Farmyard manure contains various calcium salts derived from the vegetable matter out of which it has been formed, sometimes in their original form, but partly broken down into the undefined carbon compounds known as "humates." Calcium humate, Wollny* has already shown, can be converted into calcium carbonate by bacteria present in the soil, while the following experiments show that the commonest of all calcium salts in the plant, the widely distributed calcium oxalate, is readily fermented to carbonate. 100 c.c. of a nutrient solution containing—

| | |
|---|---|
| Ammonium sulphate ............... | 0·2 gramme. |
| Sodium chloride ..................... | 0·2 " |
| Potassium hydrogen phosphate ... | 0·1 " |
| Magnesium sulphate ............... | 0·05 " |
| Ferrous sulphate ...................... | 0·04 " |

were placed in an Erlenmeyer flask plugged with cotton-wool in the usual way; to this 1 gramme of calcium oxalate was added, together with, in some cases, a small quantity of other organic nutrient, and the flask and its contents were sterilised; when cool they were seeded with 0·2 gramme of partly dried surface soil recently drawn from Plot 2 on Broadbalk Field, and the flasks placed in a dark cupboard at the ordinary laboratory temperature. The results are summarised in Table XV, and serve to show that the soil

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* 'Zersetzung der organischen Stoffe,' 1897, p. 217.

XV.—Bacterial Decomposition of Calcium Oxalate.

| Reference No. | Added to nutrient solution at starting. | Duration, days. | $CaCo_3$ found from 1 gramme $CaC_2O_4$. | Reaction at end. | State of nitrogen compounds at end. |
|---|---|---|---|---|---|
| 46 | No soil added ......... | 79 | — | — | |
| 50 | No calcium oxalate ... | 79 | 0·015 | Neutral | |
| 61 | „ „ ... | 176 | trace | „ | Strong nitrite, slight nitrate. Ammonia. |
| 44 | Neutral .................. | 79 | 0·263 | „ | Nitrate, no nitrite nor ammonia. |
| 62 | „ .................. | 176 | 0·316 | „ | Nitrate, slight nitrite, no ammonia. |
| 43 | Slightly alkaline ...... | 73 | 0·428 | „ | Strong nitrite, nitrate. |
| 64 | „ „ ... | 176 | 0·274 | „ | Slight nitrite, strong nitrate, no ammonia. |
| 42 | +0·2 glucose, neutral | 79 | 0·105 | „ | No nitrite nor nitrate. |
| 58 | +0·2 „ „ | 78 | 0·052 | „ | No nitrite nor nitrate. Ammonia. Glucose gone. |
| 52 | +0·2 „ alkaline | 73 | 0·184 | „ | Little nitrite, no nitrate, no glucose nor ammonia. |
| 48 | +0·2 peptone ......... | 80 | 0·183 | „ | Nitrite, strong nitrate, no ammonia nor organic matter. |
| 59 | +0·2 „ ......... | 84 | 0·184 | „ | Strong nitrite and nitrate, some ammonia, no peptone. |
| 47 | +0·2 calcium humate | 82 | 0·182 | „ | Nitrite and nitrate, no ammonia. |
| 60 | +0·2 „ „ | 176 | 0·195 | „ | Slight nitrite, strong nitrate, no ammonia. |
| 63 | +0·2 „ „ | 176 | 0·144 | „ | Slight nitrite, strong nitrate, no ammonia. |

contains one or more organisms which are very effective in converting calcium oxalate into carbonate. The mechanism of the reaction is being further studied; in the present connection the experiments are sufficient to show the existence of other agencies of a bacterial nature engaged in restoring calcium carbonate to the soil.

The destruction of nitrates by bacterial action, with the evolution of the nitrogen as gas, the change commonly known as "denitrification," is always attended by the production of a carbonate of the base with which the nitric acid was combined, but as any calcium carbonate formed in this way would only replace the calcium carbonate consumed in the previous nitrification there would be neither gain nor loss to the soil. As also denitrification is most likely to take place in the lower subsoil where the oxygen of the soil gases has been exhausted, any calcium carbonate re-formed in this way would not appear in the analyses set out, which only extend to the depth of 27 inches.

It is, however, clear that manuring with organic manures, the growth of clover and other leguminous plants which leave behind a considerable residue of roots and stubble particularly rich in calcium oxalate, the *débris* of plant tissues which accumulates in the soil of grass land, all go to maintain the stock of calcium carbonate, which in its turn is being as constantly drawn upon for nitrification and for the neutralisation of the other acids produced during the bacterial decay of the carbon compounds the soil receives.

Doubtless in all soils containing only a minimal amount of calcium carbonate under natural conditions these various actions have reached an equilibrium, since the increase of any one only tends to bring into play the factor which limits it (the rate of nitrification, for example, will be slowed down as the available base in the soil becomes scarce), but also accelerates the operation of some action in the opposite sense; even the one irrevocable loss by drainage and removal of crop will probably be balanced by the calcium salts coming into solution through the continued weathering of the soil particles. In the main, however, the original stock of calcium carbonate in the soil circulates continually between plant and soil without suffering appreciable loss. It is only under particular conditions, such as the use of ammoniacal manures, or the setting up of anaërobic conditions through lack of drainage, thus allowing the formation of organic decay acids but not their final oxidation to carbonates, that the soil will develop an acid reaction and become infertile.

## *Summary.*

The chief points brought out in the course of the investigation are as follows:—

(1) Arable soils which contain upwards of 1 per cent. of calcium carbonate are subject to a normal loss of that constituent in the drainage water amounting to about 800 lbs. to 1000 lbs. per acre per annum.

(2) The loss is increased by the use of ammoniacal manures by an amount equivalent to the combined acid of the manure. The loss is diminished by the use of sodium nitrate or organic *débris* like farmyard manure.

(3) The growth of plants normally returns to the soil a large proportion of the bases in the neutral salts which the soil provides for the nutrition of plants.

(4) The calcium oxalate and other organic salts of calcium present in

plant residues are converted by bacterial action in the soil into calcium carbonate.

(5) The return of base by the growth of plants and the production of calcium carbonate by the decay of plant residues are sufficient to maintain soils neutral which are poor in calcium carbonate, and to replace the bases which have been consumed in nitrification and similar changes.

# *On the Origin and Life History of the Interstitial Cells of the Ovary in the Rabbit.*

By Janet E. Lane-Claypon.

(From the Physiological Laboratory, University College. Communicated by Professor E. H. Starling, F.R.S. Received June 16, 1905.)

[Plate 1.]

The majority of the investigators of the subject consider that the cells of the germinal epithelium arise by differentiation of the peritoneum, and become embedded in the subjacent mesoblast, there being probably a dual process, namely, the downgrowth of the cells themselves and a simultaneous upgrowth of the subjacent mesoblast.

The fate of the cells thus embedded has given rise to much discussion. All observers agree in stating that they give rise to the ova, and most observers consider that they give rise also to the follicle cells; but de Foulis (8),* Schrön (16), and Wendeler (20), believe these cells to be derived from the connective tissue.

Pflüger (15) and Waldeyer (18), although differing in regard to the development of the ovary, both consider that the germinal cells give rise to the cells of the follicular epithelium, there being most probably a previous division of the original cells.

Nagel (14) also agrees that the follicle cells are derived from the germinal epithelium. Balfour (4) believed that some of the cells of the egg-clusters became ova by differentiation, and he described besides a number of small cells, of which some formed the follicular epithelium and the others probably either served as foodstuff for the rest, or eventually themselves formed ova or follicle cells.

Bühler (7) describes the formation of the follicular epithelium by the streaming inwards from the periphery of some of the cells of the germinal epithelium.

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* These numbers refer to the entries in the bibliography at end.

The changes connected with ovogenesis have been very fully described by v. Winiwarter (19). They may be briefly summarised as follows. The germinal cells of the second invagination are rather small and show a nucleus with some lumps of chromatin, being also rather granular. These he calls *protobroque* cells of the *a* variety. These divide, giving rise to other protobroque cells *a*, and also to a *b* variety. These last divide again giving rise to more cells of the *b* variety and to a new form of cell, *deutobroque*. The last are larger, and the nucleus more transparent. The deutobroque cells give rise to the ova by nuclear differentiation by means of the following stages. 1. The chromatin breaks up into fine filaments, which are distributed over the whole nuclear area; this is the *leptotenic* stage. 2. The filaments become gradually massed together until they show as a compact lump at one side of the nuclear area. This transformation is the *synaptenic* stage, which is succeeded by 3, the *pachytenic*. Here the filaments become again spread out, but they are much coarser than in the previous stages The 4th stage, or *diplotenic*, is so called on account of the tendency of the chromatin strands to lie in pairs. In the final or *dictyate* condition the chromatin is distributed in a reticulum over the greater part of the nuclear area

Balfour describes protoplasmic masses of young ova where the cells appear fused, and he suggests that one of these ova may grow at the expense of the rest. Van Beneden (5) describes multinucleated masses in the ovary of the adult bat, which he suggests may give rise to an ovum and its follicular epithelium.

The formation of follicles, which proceeds rapidly, gives rise in the ovary to two zones, an external or parenchymatous zone in which the follicles lie, and an interstitial vascular zone; these have been described with some modifications by various workers and for different animals. (His (11), Waldeyer, Born (6), Macleod (13), Van Beneden.)

The question of the post-natal formation of primordial ova has been the subject of many isolated observations. Pflüger believed he had evidence of the return of the ovary to the tubular formation at the rutting season, the object of the return being the formation of fresh ova. Waldeyer believed that all ova were formed in the young animal, and for this reason called all ova "primordial ova."

Schrön noticed an increase in the number of clear cells, presumably ova, near the periphery in cats and rabbits at the rutting season, and in women at the menstrual periods. Koster (12) describes prolongations of epithelium with formation of fresh ova and follicles in the ovaries of several recently pregnant women; Wagener (17) records the thickening of the germinal epithelium near the attachment of the Fallopian tube in the pregnant bitch, a condition which he thinks denotes the formation of ova.

Amann (1) describes the presence of follicles in the ovaries of a woman of 63, where there was incipient cystadenoma, the follicles being in all stages of formation by means of invagination of the germinal epithelium. The interest of this observation lies in the age of the woman and in the apparent formation of fresh ova consequent on the stimulus caused by the incipient cystadenoma.

V. Winiwarter was not able to trace any of the stages of ovogenesis in any of the adult ovaries he examined, and considers this a necessary condition for the formation of ova. As far as the literature goes, we may consider the ovary to be formed by the embedding in the underlying mesoblast of the cells of the germinal epithelium, the embedding being brought about by a process of ingrowth of the cells and of upgrowth of the mesoblast. The cells thus embedded are oogonia, which give rise to ova by division, as also to the follicle cells, the future ova undergoing considerable nuclear transformation before reaching the condition of the fully-formed primordial ovum.

The post-natal formation of primordial ova has been recorded in certain cases, but there is not much evidence either in favour of or against it.

*Object of the Investigations.*—Certain features which I observed in the interstitial cells of the ovaries of rabbits at a late period of pregnancy led me to study the origin of these cells. This question would appear to have been neglected by previous workers on the ovary. The formation of an internal ovarian secretion (*cf.* Andrews (3)), which by analogy with the interstitial gland of the testis might be presumed to be derived from the interstitial cells (*cf.* Ancel and Bouin (2)), gives considerable interest to their origin.

This was studied by examining (1) the ovaries of rabbits from the twentieth day embryo up to those of the young rabbit about three weeks after birth; (2) the ovaries of pregnant rabbits at all stages.

*Methods.*—It is not easy to find a really good fixing agent for ovaries, especially adult ovaries. Hermann's, Flemming's (strong formula), Podwyssoski's and Altmann's fluids, were all used. The last was found satisfactory for cytoplasm, but the sections obtained with the other fixatives were not good. The tissue was osmicated outside and insufficiently fixed inside. Finally,* Gilson's fluid was used exclusively for all nuclear figures, and a mixture of sublimate (saturated) 4 pints, formol 1 pint, and 1 per cent. glacial acetic for other purposes.

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* Gilson's fluid = abs. alc., 1 part;
glacial acetic, 1 part;
chloroform, 1 part;
the whole saturated with sublimate.

The sections fixed in Gilson's fluid were stained with iron hæmatoxylin, or hæmalum; those fixed in the other solution stain well either with iron hæmatoxylin, hæmalum and eosin, or toluidine blue and eosin.

### *Changes in the Cells of the Germinal Epithelium in the Immature Rabbit.*

The origin of the germinal epithelium from the peritoneum by a process of differentiation has been so fully shown by several observers, that it will not be necessary to deal any further with the origin of the germinal cells. Also it has been shown that these cells become embedded in the underlying mesoblast; this state of affairs is seen in an embryo of the twentieth day.

The ovary is by this time a definite organ; it is intensely vascular, showing large blood spaces, especially in the parts lying immediately round the mesoblastic core. At this period the main mass of the germinal cells is situated peripherally, only a few isolated ones having penetrated into the core, which last is sending processes of connective tissue in between groups of germinal cells. Of these there are present a large number of protobroque and a few deutobroque; also a certain number of mitotic figures, but these are not numerous. (See Plate 1, fig. 1.)

From this time onward until after birth the changes in the ovary, as seen under the low power of the microscope, are not striking; there are more deutobroque cells, characterised by their transparent appearance, and there is an increase in the number of mitotic figures.

Studied under the high power of the microscope some of the deutobroque cells are seen to have entered upon the early stages of ovogenesis, and to have reached the leptotenic stage. There are large numbers of round cells showing a nuclear structure differing from either the protobroque or the deutobroque cells. The mitoses are chiefly found near the periphery, and the greater number of them seem to be taking place in the large cells. I do not altogether agree with v. Winiwarter on the question of mitosis in these cells. In the first place there appears to be very little distinction between the varieties of protobroque cell, *a* and *b*, and I shall not dwell upon it. The mitosis in the protobroque cells does not appear to be sufficient to account for the large number of deutobroque cells which are formed, and my observations are to the effect that by far the greater number of mitoses are taking place in the deutobroque cells themselves. Each class of cell divides, the protobroque less copiously than the deutobroque, giving rise to two cells of their own variety. There can be no doubt that the deutobroque cells are modified germinal cells, but I hope to show that the process is one of differentiation, and not of division.

The protobroque cell is the type of the original germinal cell; it is small, generally oblong or oval, and contains a large nucleus. The nucleus shows a number of chromatin masses of varying sizes, and the whole nuclear area gives a general impression of granulation (represented by shading in the figures (Plate 1, fig. 2 (*a*)). There are no chromatin filaments.

The deutobroque cell is very much larger, and the nucleus has for the most part a strikingly transparent aspect, the granular appearance noticed in the protobroque cell being confined to the periphery of the nuclear area. The chromatin is quite differently arranged; there are one or two irregular chromatin masses, and strands showing nodular enlargements where they intersect. See fig. 2 (*f*).

Sections of a young ovary very soon before or after birth show a large number of cells whose nuclei exhibit every phase of transition between these two varieties. These changes in the nucleus may be classed broadly into three divisions:—

1. The chromatin masses become fewer and larger.
2. There is considerable formation of chromatin strands.
3. The granular appearance gradually passes away from the centre of the nucleus towards the periphery, leaving the centre clear.

Some of these changes are shown in fig. 2 (*b*), (*c*), (*d*), (*e*). In the first stage the whole cell becomes rounder, as also the nucleus, and the chromatin has begun to aggregate, and there are traces of strands passing away from the masses. These features increase in intensity until there are only a few chromatin masses, but the strands are passing between them and intersect in parts. The granular appearance has begun to leave the centre, which is clear.

A further process on these lines brings the cell into the typical deutobroque condition. It would therefore seem that the change from the protobroque type is accomplished by means of transformations in the nuclear area, accompanied by a growth in size of the cell, and it is unnecessary to suppose, under these circumstances, that mitosis is also a method of formation. The protobroque and deutobroque cells are therefore all oogonia, either potential or actual, the transition from the one class to the other being probably accomplished by processes of nuclear differentiation.

At this period in the life of the ovary there is no appearance which could be characterised either as egg-tubules or egg-clusters; there are large collections of epithelial cells bounded centrally by the mesoblast, which presents the appearance of connective tissue. This tissue penetrates but slightly into the region lying peripherally to the main central core, but careful inspection shows that there are a few fine processes pushing their way outwards and more or less enclosing large numbers of germinal cells. The latter are of all shapes and sizes, from the typical protobroque to the deutobroque type.

By the third day after birth the general configuration of the ovary has changed very considerably. There is still the central mesoblastic core, but the germinal cells have become more marked off than in the embryo, presenting the appearance of a definite zone of germinal epithelial cells. The cells are arranged, especially in the more central parts of the zone, in the form of solid rods or clusters, several cells thick, which press their rounded ends into the central mesoblast. Some of them might fairly easily be mistaken for tubules without a lumen, and there can I think be little doubt that these are what Pflüger took for tubules. Around the periphery the tubular formation is not so marked, the cells lying in irregular aggregations (fig. 3). As the mesoblast is centrally situated those parts of the germinal zone lying towards the centre get divided up earlier than the more peripheral parts, which retain the formation of an earlier stage. This lagging behind, as it were, of the periphery is quite characteristic of all the changes taking place in the young ovary; it applies to the formation of tubules and clusters, to the processes of ovogenesis, as pointed out by v. Winiwarter, as well as to the formation of interstitia cells, which will be dwelt upon later on.

From this time onwards up to about the twelfth day after birth the changes in the general configuration of the ovary are brought about by an amplification of the processes already described, namely, continued upgrowths of connective tissue, cutting off the tubules and clusters. The connective tissue likewise presses into the larger collections of germinal cells, thus cutting them off and dividing them again into smaller portions, so that as time goes on the clusters near the central parts consist of less cells, but are present in much greater number, while those parts more peripherally situated are in a somewhat earlier stage, the cluster formation being still fairly evident close to the periphery as late as the sixteenth day.*

Before proceeding to the changes in the egg-clusters about the fifteenth day, the transformations which have been taking place in the deutobroque cells must be briefly touched upon, but they have been so fully described by v. Winiwarter that a lengthy exposition is quite unnecessary. I shall adopt his nomenclature throughout. It has already been stated that almost immediately after birth changes begin to take place in the deutobroque cells, which enter upon the leptotenic phase, the transformations beginning centrally. This is succeeded by the synaptenic, and by the third day there are already a very great number of this variety.

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* Too much stress, however, should not be laid upon the exact date of the young ovary in relation to its structural aspect. There seems to be an appreciable difference in the extent to which the ovary is developed in different animals about this age. v. Winiwarter does not describe any ovary between the tenth and the eighteenth day, because he does not consider the changes to be sufficiently striking to call for any description. Of two litters of rabbits I found slight differences in the ovaries of the same date, the sixteenth day, the changes being rather more advanced in one than in the other, and both were almost as advanced as v. Winiwarter's figure of the eighteenth day. The differences are probably determined by the varying nutrition of the animal, as also possibly by the kind of rabbit, some being far more advanced in outward aspect at this age than others.

The leptotenic phase is characterised by the absence of visible nucleolus, and by the spreading out of the chromatin in the form of fine filaments over the whole nuclear area. This stage is evidently only a further step in the differentiation which has already taken place. It has been shown that the change from the protobroque to the deutobroque type is accomplished by the chromatin masses becoming gradually broken up into strands. In the leptotenic phase the process is merely carried further. Whereas in the deutobroque stage there are still one or two chromatin masses which have not become broken up into strands, in the leptotenic this is not the case, the whole chromatin being present in the form of filaments. The transition stage can be seen in an ovary of a few days after birth (fig. 6 (1)). These filaments become gradually aggregated, passing to the synaptenic state. The leptotenic condition is a very fugitive one, whereas the synaptenic, owing presumably to the great variety of aspects through which the aggregation passes is very much more prolonged. The synaptenic is succeeded by the pachytenic, where the filaments are coarser, then by the diplotenic, and this in its turn by the dictyate condition, which is the typical nuclear appearance of the young ovum.

These changes pass gradually outwards, and by the tenth day even the cells quite at the periphery have passed through the earliest phases, whilst the central cells are reaching the final ones. By the fourteenth day there are a certain number of dictyate nuclei towards the centre. At this stage the central mesoblastic core is becoming obliterated, the egg-clusters of either side of the ovary very nearly meeting. The clusters are much smaller, having been split up by the ingrowing mesoblast. The number of dictyate nuclei now increases at a surprising rate, there being a great number by the fifteenth day, and a still greater number by the sixteenth day, by which time the clusters are almost indistinguishable, except round the periphery, their place having been taken by dictyate cells, some of which now show a surrounding follicular epithelium. There are also collections of small more or less rounded cells lying in between the young follicles, but not forming any part of the follicular epithelium. These are the interstitial cells of the ovary, and I propose now to trace their origin in detail.

*Origin of Interstitial Cells.*—By about the tenth day the ovogenetic processes in the central egg-clusters are at their height, and continue in this condition, passing through the various phases, until about the fourteenth day, when, as already stated, some have reached their final stage. Throughout the whole period, however, there are in almost all the clusters some three or four cells, or perhaps more, which remain in the ordinary deutobroque state, and do not undergo any of the ovogenetic phases, the number of these being greater in the peripheral clusters than in the central ones; by the fifteenth and sixteenth days, when the number of dictyate nuclei is increasing, there are few, if any, of the deutobroque cells to be found in the central parts, but instead, there is an increasing number of the small round cells already referred to. As the days pass on the number of the former decreases, and the number of smaller cells increases. Thus, there is throughout the ovary, but in different parts at slightly different periods, a reduction in the number of deutobroque cells, which have remained unchanged, and an increase in the number of small round cells.

Examination of sections of the fourteenth, fifteenth, and sixteenth days near the centre of the ovary, leaves no room for doubt that some of these cells form the follicular epithelium, gradually passing towards and arranging themselves around the young ova. At the fourteenth day the number of these cells to be found near the centre is not nearly sufficient to form the follicular epithelium for the large number of young ova, while near the periphery there are many more than would appear to be necessary for the requirements of this part. By the fifteenth day the number of these cells near the centre has increased very largely, still more so by the sixteenth day, by which time many of the young ova are surrounded with follicle cells, and there are also the collections of these cells already referred to. Their number has meanwhile diminished somewhat at the periphery. The appearance in the intermediate parts gives the key to the whole question. Here are seen large numbers of these cells streaming inwards from the periphery and making their way between the egg-clusters of the periphery towards the centre, where the cluster formation can now be scarcely recognised. Here they arrange themselves around the young ova, or pass into little groups by themselves. These groups are the first beginnings of the real interstitial tissue of the ovary, and mark the commencement of the adult aspect of the organ.

There are thus two main points to be emphasised at this period in the life of the ovary. First, the passage inwards of a large number of cells from the periphery, and secondly, the commencement of the adult formation by the formation of young follicles, and the appearance of interstitial cells.

This passage inwards of cells from the periphery was noticed by Bühler; he realised that the number of small cells near the centre during the height of ovogenesis was not enough to provide a follicular covering for all the young ova which were there, and he describes the streaming inwards of the cells from the periphery, and their passage to the young ova, around which they arranged themselves, and formed the follicular epithelium.

Balfour noticed that in the later periods of ovogenesis there were present too many of the small cells, like the follicle cells, for them all to become arranged around the ova and give rise to the follicular epithelium. He was at a loss to account for the destiny of these supernumerary cells, and supposed that they must either eventually become ova or follicle cells, or be used up as food-stuffs for the other cells.

It seems to me, however, that these cells, supernumerary as far as the follicular epithelium is concerned, are in reality very important. They form the groups which represent the interstitial tissue of the fully-formed ovary, and thus, far from being unimportant, are absolutely essential for the performance of the functions of the ovary.

The question which now arises is, where do these cells come from, and what is their history of formation? It has already been indicated that the number of unchanged deutobroque cells varies inversely with the number of these cells, since these last are greater in number in the region where there are most deutobroque cells present, namely, at the periphery, especially in the region of the poles, and the high power of the microscope reveals the fact that these cells are indeed metamorphosed deutobroque cells.

The ordinary deutobroque cell presents one or two irregular chromatin masses, from which pass out filaments of varying degrees of coarseness and fineness with nodules at their intersections. The centre of the nucleus is clear, whilst around the periphery is the granular appearance already described. See fig. 2 (*f*). In the ovary of about the eighteenth day the only regions where these cells are to be seen in any appreciable numbers are round the periphery and at the poles. They stand out even under the low power on account of their general transparent aspect as compared with the surrounding cells, and also in many cases on account of their rather larger size. There are also cells whose transparency is not so great, but which show up quite markedly in contrast to the rest and are rather smaller in size than the more transparent ones. These cells are transformation stages between the deutobroque and the ordinary interstitial type, and the process resembles very much in the inverse order that which has been already described for the deutobroque formation from the protobroque.

The first stage is the gradual massing of the chromatin into irregular masses and the thinning of the chromatin strands, which become rather less in number, as do also the nodules (fig. 4 (*c*)). At the same time the granular appearance extends gradually towards the centre, although it is not until quite a late stage that it reaches the centre itself (fig. 4 (*f*) and (*g*)). The size of the cell becomes gradually less, and the amount of protoplasm relatively greater.

The retraction, as it were, of the filaments and strands towards the chromatin masses is very much more marked in the cells of smaller size, where there is a tendency for the masses to pass towards the periphery, leaving the centre clear (fig. 4 (*d*) and (*e*)).

These changes continue until nearly all the chromatin is massed, the masses becoming rounder as the process goes on. There are always traces of strand formation left, in contrast to the protobroque nucleus, where it is markedly absent. Thus the small cell derived by differentiation from the deutobroque cell does not return to the characteristic protobroque type, but shows traces of its intervening deutobroque condition in the shape of strands of chromatin, and nodules on the strands. See fig. 4 (*g*).

The cells, once reduced in size, become true ovarian cells, and may either function as follicle cells or as interstitial cells (fig. 4 (*h*) and (*k*)).

Thus we find the following processes taking place in connection with the formation of the mature ovary. The cells of the germinal epithelium become embedded in the underlying mesoblast, and, once there, may either undergo differentiation, or apparently may remain in the protobroque condition. If the former be its fate, it must undergo nuclear transformation, together with growth in size, until it reaches the deutobroque stage. Arrived at this condition it probably divides, although possibly this is not an essential, and the two cells formed by this division are of the same type. There are now two courses open for the cells thus produced; they may undergo the nuclear transformations of ovogenesis, and become primordial ova, or they may rest for a time, and finally undergo regressive transformations, becoming either follicle cells or interstitial cells. Every cell of the germinal epithelium is probably a potential ovum, relatively very few remaining in the protobroque state, although some may still be seen at the periphery in ovaries of the eighteenth day. Incomparably the greater number pass to the deutobroque state, preparatory doubtless to the formation of ova. All cannot become ova, for the other forms of cell are necessary for the maintenance of the ovarian functions; possibly, therefore, only the most robust cells, and those which are most conveniently situated for obtaining nourishment undergo the ovogenetic changes. This would seem to be borne out by the fact that many more of the central cells, which are nearer their food supply, undergo ovogenesis, than of the peripheral ones. The rest of the cells which are not able, for one cause or another, to undergo these changes, appear to remain quiescent for a while, until finally they regress and pass into a condition of subserviency to the needs of those which have become ova. Both follicle cells and interstitial cells are, however, still potential ova. They have passed through the initial stages, and only need enlargement and nuclear transformations in order to become ova, should the appropriate stimulus be given. This chance is not given to the follicle cells. As soon as the follicle begins to grow, they multiply rapidly, and probably provide, by their disintegration, the follicular secretion upon which the ovum feeds and grows. In the ripe follicle of the rabbit there is almost complete disintegration of the membrana granulosa, and the remains of the discus proligerus is presumably extruded with the ovum, perhaps serving it as food material prior to its fertilisation, and subsequent attachment to the uterine wall. The interstitial cells, however, have possibilities before them, being still capable of carrying out any function belonging to the true ovarian cell.

All the true ovarian tissue is derived from the germinal epithelium, this tissue forming in the adult rabbit by far the greatest part of the whole ovary. There is relatively little mesoblast, which subserves solely the function of support and of nutriment-carrier to the rest of the organ. We may, therefore, look upon the whole ovary as consisting of two classes of cells and of two only, namely, (1) those derived from the germinal epithelium and performing all the ovarian functions, and (2) those derived from the original mesoblast, which are supporting and vascular.

There remains only one feature to be dealt with in the immature ovary, one that has already been described by Balfour, namely, the protoplasmic masses formed by the aggregations of young ova. In the ovary of the sixteenth day the ova are all separate, but a day or two later this is not the case. There are now a large number of these masses of various sizes. They appear to consist of two, three, four, or even five young ova, to judge by the number of nuclei seen, but it is impossible to distinguish any trace of cell-boundary between them. Balfour suggests that these may either form as many ova as there are nuclei, or that one ovum may develop at the expense of the rest. This last point of view appears to be the more probable. It is evident that the massing takes place subsequent to the formation of the young ova, since it is not seen until after the appearance of the ova, and it would appear rather purposeless if they merely separated again a little later on. Moreover, in these masses one or two of the nuclei often look as if they were disappearing by gradual dissolution, and it is, therefore, probable that they will all ultimately serve as food-stuff for the one ovum whose condition happens to have been best, and will, therefore, survive in the struggle for existence.

This cannibalism on the part of the young ovum is not surprising, if the life of an ovum be considered. It is really but the normal condition of the cell at all its stages of development; it grows and fattens at the expense of other cells. In the young ovary it is starting its first stage of growth and must devour other cells; later on, when it grows during the growth of the follicle, it lives upon the follicle cells, and later still, when, after fertilisation, the ovum in its extended sense refers to the young fœtus, it lives on the material provided by the cells of the maternal organism.

This massing of cells and subsequent demolition of some of them for the benefit of one will be again dealt with in connection with the ovary of the pregnant rabbit.

### *Changes in the Ovary during Pregnancy.*

The young ovary, after the period when it has reached a stage where the general aspect is that of an adult ovary, enters upon a period of slow growth, during which there is a continual formation of a considerable number of follicles, which having reached a state of partial maturity then begin to atrophy and finally disappear, leaving only a faint trace of their former existence in the shape of a scar.

Having reached sexual maturity, the ovary becomes subject to periodic influences, of the nature of which little, if anything, is known. According to Fraenckel (9), they are intimately connected with the hypertrophy of the mucous membrane of the uterus. The sum total of the influences at work results in the production of "heat," which occurs in the rabbit about once a fortnight, but the external changes in the vulva by which this is judged take place very gradually, so much so, that in the spring and summer time, when breeding is most prolific, the adult rabbit is scarcely ever out of one or other stage of "heat." It is fairly certain, therefore, that whatever changes may take place in the ovary during "heat," the condition recurs too frequently for these to be very marked. This does not refer in any way to the formation of the corpora lutea of "heat," which are, of course, very definite. It has recently been stated by Heape (10) that unless impregnation occurs the ripe follicles of "heat" do not burst, in which case, presumably, there can be no formation of corpora lutea. If this is the case it would seem that there can be no such thing as the corpus luteum of "heat," and the changes in the ovary during this period must be considered to consist merely of those taking place before sexual maturity, only rather more marked, namely, the formation of follicles, but after puberty these reach the ripe state, since they will burst if impregnation occur, whereas this is probably not the case in the immature ovary. There would then, on this view of the case, be no ovulation except in the impregnated rabbit. It is quite possible that the additional stimulus of impregnation may hasten the bursting of the follicle, but it seems somewhat unlikely that without impregnation there should never be ovulation.

The changes resulting in the production of "heat" are obviously those preparatory to a possible pregnancy. Fertilisation appears to be in itself a stimulus, and sets up general hypertrophy of the entire genital apparatus, producing likewise an improved condition of the animal; as to the mechanism of the production of this hypertrophy, however, our knowledge may be said to be nil, and we are reduced to classifying the whole as the changes brought about by the stimulus of pregnancy.

*Naked Eye Changes.*—Fraenckel describes and figures very accurately the naked eye changes in the pregnant ovary of about the fifteenth day in his paper on the function of the corpus luteum.

These changes are very striking, and indicate in themselves some very definite alteration or increase in the function of the gland; apart from the formation of the corpora lutea, there is an immense increase in absolute size, the gradual occurrence of which will now be described.

The ovary of the non-pregnant rabbit is a small yellowish body, lying on either side against the posterior abdominal wall, a little below the kidney. It is usually about ½ inch in length and thin, being slightly wedge-shaped in transverse section and rather pointed longitudinally at either end; upon its surface may be seen clear round spots, showing the locality of the larger follicles, some of which, if they are nearly ripe, may even project slightly from the surface.

The bursting of the follicles and fertilisation lead to the formation of the corpora lutea, the so-called "true" corpora lutea of pregnancy, and the growth of these bodies during the early period are undoubtedly the most characteristic feature in the naked eye appearance of the ovary. If the pregnancy be one with a large number of fœtuses, the ovary often looks gnarled, so large and numerous are the excrescences produced upon its surface by these bodies. If these, however, be cut off, and if the organ be carefully examined at about the fourteenth day, when the corpora lutea are at their maximum state of development,* it will be readily seen that the ovary itself has increased in size, quite apart from the formation of the lutein tissue. The whole gland has a more swollen and rather less compact aspect; it is larger both in length and girth, and the wedge-shape of the transverse section is less marked; there are also in many cases fewer follicles in an advanced condition than in the non-pregnant state. Just at this period the energies of the gland have apparently been directed rather to the formation of the lutein tissue than new follicles.

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* *Cf.* Fraenckel, *loc. cit.*

From the fourteenth to the eighteenth day the corpora lutea remain at their maximum, and then begin to diminish rapidly in size. Instead of being very vascular whitish bodies, projecting in many cases to the extent of three-quarters of their whole extent beyond the surface of the gland, they gradually diminish both in size and vascularity, until by about the twenty-second day of pregnancy they are merely elevations on the surface, showing the faintest possible trace of vascularity; this diminution continues steadily until, a little while before birth, the locality of these striking features of the fourteenth day of pregnancy is only seen by the presence of an opaque whitish circular area upon the surface of the ovary. The changes are so marked that it is possible after a little experience to diagnose very approximately the previous duration of the pregnancy from the appearance of the corpora lutea.

Whilst these external changes are taking place in the lutein tissue, the rest of the ovarian tissue has been also undergoing changes, which, if not so striking in appearance, are none the less evident. It has already been stated that the organ at the fourteenth day shows marked increase in size apart from the corpora lutea; whereas, shortly after this period, these bodies begin to diminish in size, the reverse takes place in the rest of the ovarian tissue; and whereas growth of the ovary as a whole has been slow up to the present, it now becomes rapid and continues until close upon the time of parturition.

By about the eighteenth or twentieth day all trace of wedge-shape in cross-section has completely gone, and the organ is nearly circular, the girth is much greater, and this increase extends right up to the poles. These changes become more and more marked, until at about the twenty-sixth day the organ is well over an inch in length, sometimes about 1½ inches, showing a proportionate increase in its other measurements, and having a shape very much resembling a spindle with blunted ends. The number of clear round spots has meanwhile been increasing rapidly, so that in the majority of cases the greater part of the surface is taken up either by them or by the round whitish patches, which mark the spots where the corpora lutea have been projecting above the surface. The formation of follicles appears to be somewhat inhibited during the rapid growth of the corpora lutea, but to be resumed with greater energy when these have reached their maximum development. At the time of parturition there are a large number of follicles which have almost reached full maturity, and it is a well-known fact that rabbits can be readily fertilised immediately after parturition.

The gland, although soft, is not in any way brittle, and in spite of its great general enlargement retains on the whole the same shape, the most marked change being that from the wedge-shaped to the circular transverse section.

*Changes in Size of the Interstitial Cells.*—This great increase in size must be the result either of a large numerical increase, or of a very great increase in the size of the individual ovarian cells. The latter is at any rate the main, if not the only factor concerned, the change in size of the cell under the microscope being so marked as to attract attention even apart from any actual measurement.

The measurements were made with a micrometer eye-piece, gauged against a micrometer slide, this method being found quite sufficiently accurate for the purpose. It was not intended to record exactly the size of each individual cell, but rather by taking the measurements as accurately as possible of a large number, to find the average increase in size at different stages of pregnancy. In taking the measurements considerable selection was exercised in the cells measured; only those whose area in section was approximately circular, and where the nucleus was centrally situated being used, as it was hoped by these precautions to obtain measurements passing as nearly as possible through the centre of the cell. The measurements are given below of 10 cells from each date of pregnancy, but this does not by any means represent the number actually measured, but the same figures recur again and again, and the average works out to almost precisely that given.

Towards the end of pregnancy there is considerable difficulty in finding the right kind of cells to measure, nearly all of them being angular and irregular in outline, giving as a whole somewhat the appearance of a tesselated surface. The changes in general aspect of the sections, produced by the change in the size of the cells, will be returned to later on.

Interstitial Cells of a normal Rabbit.

| Diameter in mm. |  | Diameter at 14th day of pregnancy.<br>mm. |  |
|---|---|---|---|
| 0·0162 |  | 0·0225 |  |
| 0·0180 |  | 0·0216 |  |
| 0·0171 |  | 0·0243 |  |
| 0·0189 |  | 0·0207 |  |
| 0·0180 | Average = 0·0177 mm. | 0·0252 | Average = 0·0234 mm. |
| 0·0153 | = 17 μ | 0·0216 | = 23·4 μ |
| 0·0180 |  | 0·0225 |  |
| 0·0198 |  | 0·0270 |  |
| 0·0171 |  | 0·0252 |  |
| 0·0189 |  | 0·0243 |  |
| 0·1773 |  | 0·2349 |  |

| Diameter at 18th day.<br>mm. |  | Diameter at 20th day.<br>mm. |  |
|---|---|---|---|
| 0·0252 |  | 0·0342 |  |
| 0·0279 |  | 0·0333 |  |
| 0·0288 |  | 0·0306 |  |
| 0·0279 |  | 0·0306 |  |
| 0·0270 | Average = 0·0272 mm. | 0·0297 | Average = 0·0319 mm. |
| 0·0288 | = 27·2 μ | 0·0315 | = 31·9 μ |
| 0·0270 |  | 0·0324 |  |
| 0·0252 |  | 0·0315 |  |
| 0·0270 |  | 0·0324 |  |
| 0·0279 |  | 0·0333 |  |
| 0·2727 |  | 0·3195 |  |

| Diameter at 22nd day.<br>mm. |  | Diameter at about 26th day.<br>mm. |  |
|---|---|---|---|
| 0·0324 |  | 0·0306 |  |
| 0·0315 |  | 0·0288 |  |
| 0·0324 |  | 0·0297 |  |
| 0·0315 |  | 0·0342 |  |
| 0·0315 | Average = 0·0326 mm. | 0·0270 | Average = 0·0318 mm. |
| 0·0306 | = 32·6 μ | 0·0333 | = 31·8 μ |
| 0·0360 |  | 0·0360 |  |
| 0·0351 |  | 0·0351 |  |
| 0·0342 |  | 0·0315 |  |
| 0·0315 |  | 0·0324 |  |
| 0·3267 |  | 0·3186 |  |

| Diameter just before birth, at 28th or 29th day. mm. | | Diameter a few hours after parturition. mm. | |
|---|---|---|---|
| 0·0288 | | 0·0270 | |
| 0·0315 | | 0·0279 | |
| 0·0270 | | 0·0279 | |
| 0·0297 | | 0·0270 | |
| 0·0288 | Average = 0·0298 mm. | 0·0270 | Average = 0·027 mm. |
| 0·0315 | = 29·8 μ | 0·0261 | = 27 μ |
| 0·0288 | | 0·0252 | |
| 0·0306 | | 0·0279 | |
| 0·0315 | | 0·0270 | |
| 0·0306 | | 0·0279 | |
| 0·2988 | | 0·2709 | |

| Diameter after 3 days' lactation. mm. | | Diameter after 6 weeks' lactation. mm. | |
|---|---|---|---|
| 0·0270 | | 0·0180 | |
| 0·0252 | | 0·0171 | |
| 0·0279 | | 0·0162 | |
| 0·0270 | | 0·0180 | |
| 0·0270 | Average = 0·0268 mm. | 0·0171 | Average = 0·0171 mm. |
| 0·0252 | = 26·8 μ | 0·0153 | = 17·1 μ |
| 0·0279 | | 0·0180 | |
| 0·0261 | | 0·0171 | |
| 0·0270 | | 0·0162 | |
| 0·0279 | | 0·0180 | |
| 0·2682 | | 0·1710 | |

Tabulating these results, one gets—

| Approximate age of pregnancy. | Diameter of cells in μ. |
|---|---|
| 0 (= normal) | 17·0 |
| 14th day | 23·4 |
| 18th „ | 27·2 |
| 20th „ | 31·9 |
| 22nd „ | 32·6 |
| 27th „ | 31·8 |
| Shortly before birth | 29·8 |
| „ after „ | 27·0 |
| 3 days „ „ | 26·8 |
| 6 weeks „ „ | 17·1 |

Taking the radius of the cells it is seen that the increase in its length in the cell during pregnancy is from 8·5 to 16·3 or very nearly double.

If the volume of the sphere be taken as $\frac{4}{3}\pi \cdot r^3$ and the cell be taken as a sphere, the ratio of the non-pregnant cell to the cell at the maximum size attained during pregnancy becomes almost exactly 1 : 7, which would allow sufficient enlargement of the ovary to account fully for the increase in size. It is not to be supposed that all the cells enlarge to the same extent, but it may reasonably be supposed that they enlarge to about five times their normal size. This will account for the enlargement of the whole ovary, and there would seem therefore to be no necessity to seek any further cause of the enlargement of the ovary during pregnancy.

The only other possible cause which suggests itself at once is of course the division of cells, but although I have examined some hundreds of sections of pregnant ovaries, I have not found any trace of this happening. In giving the above figures I do not wish to suggest that the measurements are absolute. They are subject most probably to individual variations, depending possibly upon the number of fœtuses in each pregnancy, and on various other circumstances. The ovaries in question were, however, taken quite haphazard in regard to all external causes, which allows some scope for differences in the ovary, and the results are fairly definite. They show a great increase in the size of the ovarian interstitial cells during pregnancy, and that the main increase is reached by about the twenty-second day, and is sustained until just before birth, when there is a slight diminution in size.

In this connection there is one feature to be dealt with, namely, the shape of the cell. Up to about the twenty-fourth or twenty-fifth day it is not difficult to find approximately spherical cells to measure. After this period, however, the difficulty of doing so becomes very great, if not impossible. The cells are angular and seem crushed together, and I would suggest that possibly the cells may be really still undergoing slight increase in size, but that the capsule having almost reached its maximum stretching capacity does not admit of the desired expansion, and the cells instead of being spherical become more closely packed in order to find room for the additional bulk, filling in as it were the interstices rather than causing an increase in size in the spherical direction.

The rounded appearance is resumed very shortly after birth, and there is also a slight decrease in size. Why there should be a decrease before birth is a point upon which I feel it is impossible to offer any suggestion. The mechanism of the production of labour is a question upon which very little is definitely known; if, however, it be the function of the ovary to cause the adhesion of the fœtus to the uterine wall (Fraenckel), a function carried out presumably by means of the interstitial cells, since these probably furnish the internal secretion of the organ, it seems not impossible that the diminution in size may be indirectly connected with the onset of labour.

### *The Formation of "Primordial" Ova from the Interstitial Cells.*

In addition to the increase in size there are other changes taking place in some of the interstitial cells near the peripheral parts of the ovary, during the later third (approx.) of the period of gestation. It is a matter of common histological knowledge that over the surface of the ovary there is a layer of epithelial cells, roughly about two cells deep, although varying slightly in thickness at different places. Immediately below this is a layer of tissue in which are embedded the primordial ova in their early stages, when they have not yet acquired a follicular epithelium or when that epithelium is not very highly developed. There are in addition groups of small ovarian cells which will eventually, as occasion arises, form the follicle cells for the primordial ova. This whole layer together with the germinal epithelium varies very considerably in thickness in different animals, the variation having possibly some relation to the age of the animal under investigation (*cf.* v. Beneden).

In the non-pregnant animal and in the early periods of pregnancy, there is a fairly sharp boundary between these outer layers and the deeper lying interstitial cells. By about the twentieth day of pregnancy this state of affairs is seen to be gradually changing, and some of the interstitial cells are becoming surrounded by the connective tissue of the inner layer and thus getting cut off from their fellows below. Whether this is brought about by the passing outwards of the cells themselves or by the growth inwards of the connective tissue is very difficult to decide quite satisfactorily; but I think it is reasonable to suppose that both processes are involved. It has already been shown that there are two means whereby the germinal cells of the embryo become embedded in the subjacent mesoblast, namely, by an ingrowth of the germinal cells and by a simultaneous upgrowth of the mesoblast lying below. Here we have an analogous condition, but the positions are reversed; the germinal cells are now inside and the mesoblast outside.

This process, which is beginning to be evident about the twentieth day, continues throughout the rest of pregnancy, so that as the days go on more cells become cut off and press outwards, in many cases reaching almost to the periphery. The number of cells thus cut off varies appreciably in different animals, probably depending upon the age of the animal, but it is not excessive at any time; I have never found more than three or four rows of cells cut off, and these rows do not form continuous layers round the ovary (*vide* fig. 5).

About the twenty-third or twenty-fourth day, and in ovaries of later dates of pregnancy, a somewhat striking feature about some of these cells is that they are no longer mononucleated; two nuclei are frequent, three quite common, whilst in some cases there may be as many as six. These nuclei are not massed together as in a giant cell, but lie separate in the cell protoplasm. The latter is very much greater in amount than in an ordinary interstitial cell, and is irregular in outline. The appearance of these multinucleated cells suggests that they have been derived from the fusion of the same number of interstitial cells as there are nuclei in the cell. It will be remembered that van Beneden pointed out this appearance in the bat's ovary, when he found in some cases as many as eleven nuclei, and he suggested that possibly one of them grew at the expense of the others, whom it used as food, or that one might become an ovum and the others the follicle cells.

Examination of a large number of these cell masses shows that in many cases there is undoubted atrophy of one or more of the nuclei going on. In some there is a clear space where a nucleus might have been expected, in others the nucleus stains very faintly or only in parts, whilst there is usually one nucleus which stains intensely, especially in the iron hæmatoxylin specimens, and in which the staining, even after extreme differentiation, is still so dark as to remove all possibility of tracing any nuclear structure. This points to some difference of metabolic condition, and the conclusion seems obvious that this nucleus is growing strong at the expense of the others; one is reminded of the protoplasmic masses described by Balfour in the young ovary and to which reference has already been made in this paper. Here we have a number of potential ova (for the fact has already been emphasized that all interstitial cells being derived from the germinal epithelium are potential ova) massed together, of which the nucleus of one of them grows at the expense of the others, which it uses as food material; in the young ovary the end-product is a primordial ovum. In the pregnant ovary the end-product is likewise a "primordial" ovum. The cells of these aggregations are all quite clearly ordinary interstitial cells, and the surviving cell is also an interstitial cell differing only in the intensity of its staining reaction.

It has I hope been conclusively shown, in the earlier part of this paper, that the interstitial cells have all been derived from the cells of the germinal epithelium, and have all passed through the deutobroque condition, and it has been pointed out by v. Winiwarter that if there is to be ovogenesis subsequent to the first great ovogenetic period, the cells which are to become ova must pass through the requisite nuclear changes. Also it is obvious, although this is not a point which he brings out, that there is a very great difference in size between the primordial ovum and the interstitial cell in a non-pregnant animal, and it is therefore necessary for the cell to enlarge at some period of the transformation. This requirement is fulfilled, as has already been shown, in the case of the pregnant ovary. The interstitial cell of the non-pregnant ovary has approximately a diameter of 17 $\mu$, but increases up to 29 $\mu$ or even rather over 30 $\mu$ in the pregnant animal. The size of a primordial ovum before it begins to grow, preparatory to becoming a Graafian follicle, is very constant; I have taken measurements of a large number of ova both in the young ovary and in the pregnant as well as the non-pregnant animal, and the average diameter is 27 $\mu$, the diameter reached by the interstitial cells about the eighteenth day of pregnancy.

It is about the twentieth day of pregnancy that the cutting off of the interstitial cells towards the periphery begins to be noticeable—that is to say, shortly after they have reached a diameter about equal to that of a primordial ovum. It is not, however, until a little later that the cells thus cut off begin to show any nuclear differentiation, in fact this is perhaps best seen in the ovary of a rabbit whose time of parturition has almost arrived. These changes are identical with those taking place in the deutobroque cells of the young ovary during the period of ovogenesis. The only difference lies in the fact that whereas in the pregnant ovary the process is taking place only at the periphery, and in relatively very small numbers, in the young ovary there may be 20 or 30 nuclei undergoing changes in the same field. The fact of their presence at all in the pregnant ovary is, however, all proof that is necessary for the formation of ova. It is not for a moment to be supposed that any formation of fresh primordial ova after the first great period should take place to anything like the same extent. Probably the actual changes only occur over a period of a few days, commencing about the twenty-fifth day of pregnancy, or rather earlier, and extending probably to a little after parturition. In the young ovary the changes do not commence until after birth, and some of the cells have completed their changes by about the tenth or eleventh day, the process being probably considerably less lengthy than this for the individual cells, and taking still less time, if anything, in the pregnant rabbit, where there is obviously a state of stimulation during the whole period of pregnancy.

The first change passed through by the nucleus of an interstitial cell, which has passed to the periphery in order to become an ovum, is shown in fig. 6 (1). The nucleus shows chromatin filaments, in the middle of which are seen irregular lumps of chromatin. (In the diagrams the analogous stage of the young ovary has been given side by side with that in the adult, and does not call for any special description.) This is a transition form from the interstitial nucleus to the leptotenic stage in the process of ovogenesis, and appears to be brought about by the breaking up of the nuclear chromatin into an immense number of filaments. The arrangement of the chromatin in the interstitial cells is, as a rule, discrete either in a rather loose reticulum or round the edges, usually the former.

The first change is therefore the formation of fine filaments. The *leptotenic* stage of v. Winiwarter is brought about by the enlargement of the nuclear area and the spreading out of the filaments over this increased space, thus producing a looser arrangement which consists of fine filaments with a rather nodular appearance where they intersect (fig. 6 (2)). This state would appear to be a very fugitive one (as observed likewise by v. Winiwarter), judging by the rarity of its occurrence. It is quickly passed through, and the nucleus enters upon the *synaptenic* condition (fig. 6 (3)). This stage occupies much longer than the last, and a relatively large number of nuclei are found in this condition, which has many modifications. The filaments at the leptotenic stage are spread out over the nuclear area, whilst at the final synaptenic the chromatin is massed into a lump at the side of the nucleus. All stages may be traced both in the adult pregnant ovary and the young ovary, but only the most characteristic phase is figured, namely, that where a very appreciable amount of massing has already proceeded, the mass being connected to the sides of the nucleus by a few very fine filaments.

The massing completed, there seems to be a rearrangement of the chromatin, and it becomes spread out again, but this time the filaments are thicker. This is the *pachytenic* stage (fig. 6 (4)). The number of nuclei found in this stage is less than in the synaptenic, but still there are a fair number in various conditions. The filaments are so markedly thicker and more bulky generally that it is impossible to confuse it in any way with the leptotenic phase. The chromatin does not fill the nucleus quite so much as in the young ovary, but I have found sections where this was more the case than in the one figured; moreover in some the chromatin seems to have a more continuous disposition than is here represented.

The transition stage between the pachytenic and dictyate or final stage is not, according to my observations, quite analogous to v. Winiwarter's, and I rather hesitate to call it diplotenic, as the duality of the filaments is not well marked (*see* fig. 6 (5)); the chromatin is still arranged in thick strands, and there is some trace of nucleoli, whilst at the same time there are a very few thinner nodulated strands, foreshadowing the condition called by v. Winiwarter *dictyate*, and which represents that of the young ovum.

The nucleolus in the dictyate condition (fig. 6 (6)) is very definite, and the chromatin is arranged more or less all over the nuclear area (which is now very large), and shows a number of small nodules both at what appear to be free ends and at the points of intersection. There can, in fact, be not much doubt that the changes taking place are identical with those seen in the young ovary, which lead to ovogenesis, and therefore it would appear that ovogenesis also takes place in the adult animal during pregnancy.

Previous observers on this subject appear to have all considered that formation of ova must be accomplished by means of fresh invaginations of germinal epithelium, and those who thought they saw invaginations concluded at once that there was therefore a formation of ova in later life, whilst those who failed to find them denied the possibility on this account. My observations show that fresh invaginations of the germinal epithelium are not a necessity, but that the "invagination" has taken place already in the embryo. The invaginated cells of the germinal epithelium give rise to all the cells of the true ovarian tissue, which are all capable of functioning in any true ovarian capacity—that is, they may become ova or follicle cells, or interstitial cells, and most probably also lutein cells, their destiny appearing to be a matter of chance. The interstitial cells, however, are still capable of becoming ova, and of undergoing the changes requisite for ovogenesis should the appropriate stimulus be given. This stimulus is supplied when the animal becomes pregnant, and the ovarian cells enlarge in size. Towards the end of the time of pregnancy some of them press towards the periphery and undergo the necessary changes, becoming true ova. Thus every pregnancy would seem to be a stimulus for the next, in the way of providing new ova, although even of the relatively small number found probably very few ever reach maturity.

*Conclusions.*—Summing up the conclusions reached in this paper we find—

1. That a large number of germinal cells become embedded in the subjacent mesoblast. Of these the great majority undergo transformations up to a certain stage. This stage having been reached, they may pass through the necessary processes of ovogenesis, or they may become modified to form either follicle cells or interstitial cells, this last process being the chief fate of the cells near the periphery, whilst ovogenesis is that of the more centrally situated ones.

2. The interstitial cells are thus potential ova, capable of becoming ova should the appropriate stimulus be given.

3. This stimulus is provided by pregnancy, during which period the interstitial cells undergo enlargement in size, exceeding that of a primordial ovum.

4. About the twenty-third day some of the interstitial cells become cut off near the periphery and pass through the nuclear transformations of ovogenesis, becoming true ova.

I wish to express my deep obligation to Professor Starling, under whose supervision this research has been carried out, and without whose never-failing assistance, interest, and sympathy at each step of the work it would have been impossible to carry out the investigations described above.

Also I desire to thank Mr. H. G. Plimmer for his kindness in giving me much valuable information in regard to the carrying out of the histological details.

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## DESCRIPTION OF PLATE 1.

FIG. 1.—Ovary of 20th day embryo (rabbit). Fixed in Gilson's fluid :—

*a* = protobroque cells.
*b* = „ (in mitosis).
*c* = deutobroque cell.
*d* = connective tissue cell.
*e* = position of mesoblastic core.

FIG. 2.—Cells in ovaries of rabbits just before and after birth. Fixed in Gilson's fluid :—

*a* = protobroque cell.
*b*, *c*, *d*, *e* = transition forms between (*a*) and (*f*).
*f* = deutobroque cell.

FIG. 3.—Ovary of three days old rabbit showing formation of egg-clusters. Fixed in sublimate solution :—

*a* = protobroque cell.
*b* = deutobroque cell.
*c* = „ in mitosis.
*d* = leptotenic stage in ovogenesis.

FIG. 4.—Cells in ovaries of young rabbits. Fixed in Gilson's fluid :—

*a* = deutobroque cell in ovary of three days old rabbit.
*b* = „ „ about 18 days old rabbit.
*c*, *d*, *e*, *f*, *g* = transition stages from deutobroque to ovarian cell in ovary of about 18 days old rabbit.
*h* = interstitial cell, *k* = follicle cell } from ovary of young rabbit (18 days).

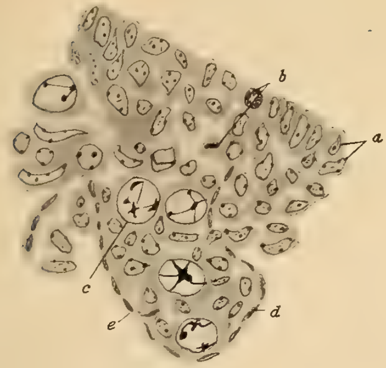

FIG. 1.

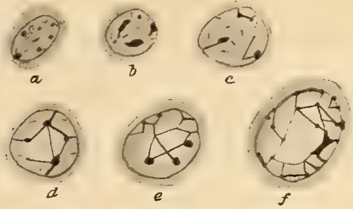

FIG. 2.

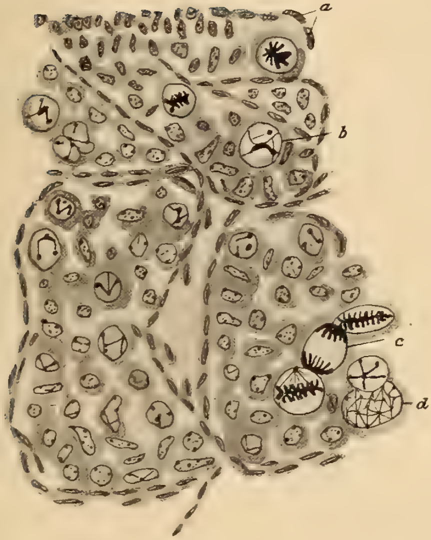

FIG. 3.

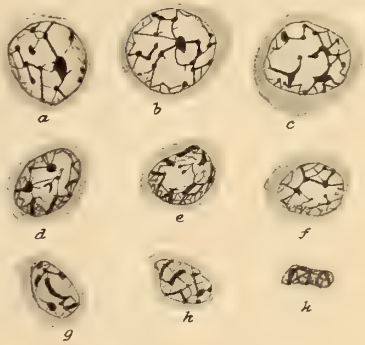

FIG. 4.

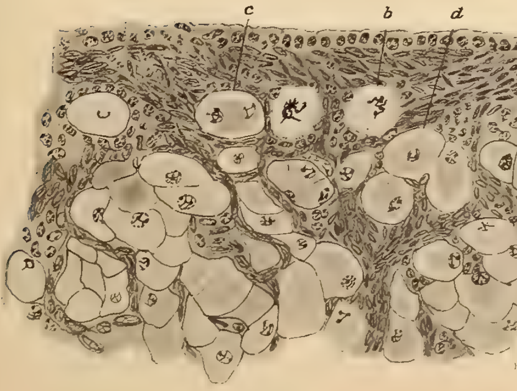

FIG. 5.

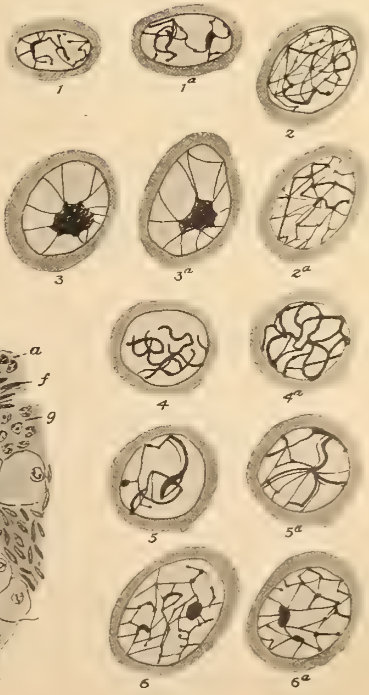
1
1a
2
3
3a
2a
4
4a
5
5a
6
6a

FIG. 6.

Fig. 5.—Ovary of rabbit about 22nd day of pregnancy. Taken from the cortical region. Fixed in sublimate solution :—

*a* = germinal epithelium.
*b* = primordial ovum.
*c* = multi-nucleated interstitial cell.
*d* = interstitial cell, becoming isolated.
*f* = connective tissue.
*g* = modified germinal cells.

Fig. 6.—1—6 are taken from the ovaries of rabbits in the later stages of pregnancy. 1*a*—6*a* from ovaries of young rabbits : showing ovogenetic changes for comparison with 1—6. Fixed in Gilson's fluid :—

1 = transition from interstitial to leptotenic phase in pregnant ovary.
1*a* = " " young "
2 = Leptotenic phase in pregnant ovary.
2*a* = " young "
3 = Synaptenic phase in pregnant ovary.
3*a* = " young "
4 = Pachytenic phase in pregnant ovary.
4*a* = " young "
5 = Diplotenic phase in pregnant ovary.
5*a* = " young "
6 = Dictyate phase in pregnant ovary.
6*a* = " young "

# *Fertility in Scottish Sheep.*

By Francis H. A. Marshall, M.A. (Cantab.), D.Sc. (Edin.), Carnegie Fellow, University of Edinburgh.

(Communicated by Professor E. A. Schäfer, F.R.S. Received August 10, 1905.)

My attention was first directed to the subject of fertility by Mr. Walter Heape, to whom I am much indebted.

Experiments have been described in agricultural publications on the effects of different methods of feeding and general treatment upon wool or meat production; but excepting, so far as I am aware, for Mr. Heape's report on "Abortion, Barrenness, and Fertility" in sheep in the South of England for the year 1896 to 1897,* no systematic attempt has been made to deal with the factors which influence fertility either in the sheep or in other animals. Numerous experiments, however, are annually conducted by flock-masters for a practical object, and it has been thought desirable to put the results of some of these on record with a view to making comparisons, and in the hope eventually of reaching definite conclusions upon this subject.

That differences in food and environment exercise an influence over fertility in the sheep as in other animals has long ago been recognised,† and recently attention has been called to the wide range of variability in the sheep's sexual capacity, this animal showing a complete gradation between the monœstrous condition and the most extreme degree of polyœstrum.‡

As a preliminary step in an investigation on fertility in sheep, it was decided to issue a schedule of queries addressed to various flock-masters chiefly in the East of Scotland. The present communication consists of a condensed account of some of the information contained in their replies.§

The preparation and issue of the schedule was undertaken by the Highland and Agricultural Society of Scotland, under whose auspices the work is being carried on. I am under no light obligation to the members of this society for their co-operation, as well as to all those gentlemen who have supplied me with information regarding their personal experiences. The comparatively small number of schedules issued and returned (the latter being about 50), rendered it possible to obtain fuller information than would otherwise have been the case, while the information obtained in this way was in some cases supplemented by personal conversation or further correspondence. For the purpose of showing the percentages of lambs per ewes, of barrenness, and of abortion (Tables I, II, and III) among flocks treated in different ways, these are divided into six groups as follows:—*

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* "Abortion, Barrenness, and Fertility in Sheep," 'Journ. Roy. Agric. Soc.,' vol. 10, 1899. "Note on the Fertility of Different Breeds of Sheep," 'Roy. Soc. Proc.,' vol. 64, 1899.

† Darwin, "Animals and Plants," Popular Edition, London, 1905.

‡ Marshall, "The Œstrous Cycle and the Formation of the Corpus Luteum in the Sheep," 'Phil. Trans.,' B, vol. 196, 1903.

§ It is hoped that a full report may be issued next year in the 'Transactions' of the Highland Society.

*Division A.*—This includes hill sheep (Scotch Black-faced and Cheviots), which were kept all the year round on the sides of hills, and received no sort of special treatment.

*Division B.*—This includes hill sheep (Scotch Black-faced and Cheviots), which were placed upon better grass at tupping time (*i.e.*, during the sexual season) or shortly before.

*Division C.*—In this division are included half-bred (Border Leicester × Cheviot) ewes which underwent a process of flushing by being fed on turnips, cabbages, oats, dried grains, maize, or other artificial food during tupping and for about three weeks before. The ewes were in most cases merely fed on grass during the greater part of the year, but received a certain amount of extra food (turnips, etc.) during the latter part of pregnancy (*i.e.*, usually from about the beginning of the year).

*Division D.*—This includes two flocks of Cheviot ewes which were flushed at tupping time but were fed on grass during the rest of the year.

Table I.—Number of Lambs per 100 Ewes.

| Flocks. | Under 90 p. c. | 90 p. c. | 100 p. c. | 110 p. c. | 120 p. c. | 130 p. c. | 140 p. c. | 150 p. c. | 160 p. c. | 170 p. c. | 180 p. c. | 190 p. c. | Total. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Division A | 2 | 10 | 2 | 1 | — | — | — | — | — | — | — | — | 15 |
| „ B | — | 1 | 1 | — | 1 | — | 1 | — | — | — | — | — | 4 |
| „ C | — | — | — | — | 1 | 1 | — | 2 | 3 | 1 | — | 2 | 10 |
| „ D | — | — | — | — | 1 | 1 | — | — | — | — | — | — | 2 |
| „ E | — | — | — | — | — | — | 1 | 1 | 3 | — | 1 | 1 | 7 |
| „ F | — | — | — | — | — | — | 1 | 1 | 2 | 2 | — | — | 6 |
| Total...... | 2 | 10 | 3 | 1 | 3 | 2 | 3 | 4 | 8 | 3 | 1 | 3 | 44 |

The numbers represent the numbers of flocks, the total being 44. The percentages are the percentages of lambs per ewes in the different flocks. The flocks are arranged in six divisions, according to the methods of feeding, as explained in the text.

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* The variation in the number of flocks in the three tables is due to the flock-masters not having supplied complete information in all cases. Some flocks, therefore, are included in one table but not in another.

Table II.—Percentage of Ewes that Aborted.

| Flocks. | None. | Under 1 p. c. | 1 p. c. | 2 p. c. | 3 p. c. | 4 p. c. | 5 p. c. | Total. |
|---|---|---|---|---|---|---|---|---|
| Division A ......... | — | — | 6 | 3 | 2 | 2 | 1 | 14 |
| „ B ......... | — | 3 | — | — | — | 1 | — | 4 |
| „ C ......... | 4 | 3 | 2 | 1 | — | — | — | 10 |
| „ D ......... | — | 1 | 1 | — | — | — | — | 2 |
| „ E ......... | 2 | 3 | 1 | 1 | — | — | — | 7 |
| „ F ......... | 2 | 1 | 1 | — | — | 1 | — | 5 |
| Total ......... | 8 | 11 | 11 | 5 | 2 | 4 | 1 | 42 |

The numbers represent the numbers of flocks, the total being 42. The percentages are the percentages of ewes which aborted in the different flocks. The flocks are arranged in six divisions, according to the methods of feeding, as explained in the text.

Table III.—Percentage of Barren Ewes.

| Flocks. | None. | Under 1 p. c. | 1 p. c. | 2 p. c. | 3 p. c. | 4 p. c. | 5 p. c. | 6 p. c. | 7 p. c. | Total. |
|---|---|---|---|---|---|---|---|---|---|---|
| Division A | — | 1 | — | 2 | 3 | — | 5 | 2 | 1 | 14 |
| „ B | — | — | — | 1 | 1 | 1 | — | 1 | — | 4 |
| „ C | 3 | — | — | 3 | 1 | 1 | — | — | — | 8 |
| „ D | — | — | 1 | 1 | — | — | — | — | — | 2 |
| „ E | — | — | 2 | 4 | — | 1 | — | — | — | 7 |
| „ F | — | — | 2 | 1 | — | 1 | 1 | — | — | 5 |
| Total...... | 3 | 1 | 5 | 12 | 5 | 4 | 6 | 3 | 1 | 40 |

The numbers represent the numbers of flocks, the total being 40. The percentages are the percentages of barren ewes in the different flocks. The flocks are arranged in six divisions, according to the methods of feeding, as explained in the text.

*Division E.*—This includes flocks of Border Leicester and half-bred (Border Leicester × Cheviot) ewes which were placed on better pasture during tupping and for some time (usually about three weeks) before, but which otherwise received no sort of special treatment; in some instances, however, the ewes received a limited number of turnips during pregnancy.

*Division F.*—This division includes Border Leicester, and half-bred (and a few Cheviot) ewes which were fed all the year round on grass, receiving no special treatment of any kind.

Table I shows very clearly that the percentage of lambs was, as a rule, larger among flocks which underwent a process of artificial stimulation during the sexual season, while Table III shows that the percentage of barren ewes was generally relatively less in such flocks. The Cheviot and Black-faced sheep in Division B which produced less than 100 lambs per 100 ewes (Table I) are stated to have been unusually unprolific owing to their never having properly recovered from the extreme cold in March and April, 1904. This case, therefore, may be regarded as exceptional. The percentage of barren ewes in this flock was six (Table III).

In the three cases in which the percentage of lambs was over 190 the exact numbers were 191·5 per cent., 193·75 per cent., and 196 per cent.

In the first of these the ewes (which were half-bred Cheviot × Border Leicester) were fed on grass only, during the previous summer. For three weeks (during tupping) they were given a full supply of turnips on grass, and between tupping and lambing (five months) they were given a mixture of dried grains and turnips, and "lamb food" for three weeks before lambing. The rams (which were pure Border Leicesters) were given bruised oats during tupping. No record was kept of the ages of the ewes. One ewe had four lambs and 12·5 per cent. had triplets.

In the second case the ewes (half-bred) were fed upon Bombay cake, bruised barley and a little linseed as well as turnips and cabbages during tupping (after grass), and some turnips were given during pregnancy. The rams (Border Leicester and Oxford Down) were similarly treated. The ewes were all three-shear. Flushing with turnips was found to bring the ewes in season very rapidly. Triplets were produced by 13·5 per cent. of the ewes.

The third case is recorded under Division E, but ought possibly to have been included under Division C. At tupping time the ewes (which were half-bred) were put upon better pasture, and between tupping and lambing they were given some turnips and as much cut hay as they would eat. Previously to tupping they were fed on grass alone.* The ewes were all ages up to four-shear. The rams (which belonged to the Border Leicester, Oxford Down and Cheviot breeds) were supplied with no artificial food at tupping.

The twins appear almost invariably to have been born early during lambing time, thus showing that the reproductive activity of the ewes is generally greatest early in the tupping season. Only two returns record that twins were mostly born late, while 28 state that early twins were the rule, both among the artificially fed flocks and those which received no special treatment.

There is abundant evidence also that flushing hastens forward the tupping time. It has recently been shown that "heat" in animals is almost certainly brought about by an internal secretion elaborated in the ovaries.† It would appear, therefore, that the artificial feeding exercises a stimulating influence

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[*] Cheviot ewes, kept on the same farm, and treated similarly, produced only 10·0 per cent. lambs.

[†] Marshall and Jolly, "Contributions to the Physiology of Mammalian Reproduction. Part II.—The Ovary as an Organ of Internal Secretion," 'Phil. Trans.,' B, vol. 198, 1905.

over the secretory activity of the ovaries, while at the same time causing the Graafian follicles to mature more rapidly and a larger number to discharge during the earlier œstrous periods in the sexual season.

Regarding the effects of artificial feeding during one tupping season upon the fertility of the sheep in after years, it has so far been difficult to obtain precise information. The opinion usually expressed is that flushing is not detrimental to subsequent fertility unless it is overdone; but in a very few of the returns the view is stated that the after-effect is adverse. It is also said that if ewes are flushed one year the process must be repeated the next: otherwise the ewes tend to be less fertile than if they had never been flushed at all.

On the other hand, several of the returns show that sheep which produce twins one year very frequently bear twins also in the year following. This seems to occur irrespectively of whether it was the practice to flush the ewes. It would appear, therefore, that an increased degree of fertility is characteristic of certain particular ewes.

That fertility is a character which can be inherited admits of no doubt. It is to be noted, however, that with the breeds considered in this paper, twins are seldom if ever selected for purposes of tupping, since they generally are not so well developed, owing to their having had less nourishment when they were young lambs. It would seem, therefore, that the fertility of these breeds is diminished owing to the fact that the rams which are probably naturally the most fertile are the ones which are the least frequently employed for breeding

# *On the Nature of the Galvanotropic Irritability of Roots.*

By Alfred J. Ewart, D.Sc., Ph.D., F.L.S., and Jessie S. Bayliss, B.Sc.

(Communicated by Francis Darwin, For. Sec. R.S. Received September 7,—
Read November 23, 1905.)

After the contradictory statements of Elfving[*] that roots curve towards the positive electrode (anodotropic), and of Muller-Hettlingen,[†] that they were kathodotropic, Brunchhorst[‡] apparently reconciled these contradictory observations by finding that strong currents, like those used by Elfving, produced a curvature to the positive electrode, weak ones a curvature to the negative electrode. The former curvature Brunchhorst considered to be traumatropic in character, on the ground that it was shown by decapitated roots, whereas the negative curvature was not. The proof that the galvanotropic irritability resides solely in the root tip, is, however, quite insufficient, and hence Brunchhorst's conclusion does not appear to be justified by the facts. The methods of the first two investigators leave much to be desired, and although Brunchhorst's experiments were, in part, carried out on a klinostat, they are by no means perfect. Thus the roots were immersed in water in a closed vessel, through which the current was passed by means of carbon electrodes. Apart from the effects due to the gases occluded by the electrodes, and to the deficiency of oxygen in the water, there would always be a tendency for the current to run obliquely or longitudinally through the roots, whose tissues form better conducting media than the surrounding water. This tendency will be especially pronounced when the roots are not exactly at right angles to the current, as is practically always the case, and when, as in Brunchhorst's experiments, numerous roots are examined at the same time. Finally, although Brunchhorst gives some data as to the total amount of current flowing in the circuit, these data afford no evidence as to the actual amount of current passing through the individual roots. Evidently, therefore, the supposed positive and negative parallelo-galvanotropism of roots is by no means satisfactorily established, and accordingly Miss Bayliss undertook to reinvestigate this subject, under more well-defined and controllable conditions, and with the results given in brief below.[§]

The strength of constant current required to produce a curvature is incredibly small, for using a voltage of approximately 1·3 volts, a resistance of 100,000 to 150,000 ohms was required in the circuit, so that the current passing through the 1 to 3 sq. mm. of cross-section lay between 0·0000135 and 0·000009 of an ampère. Even then it was difficult to produce a curvature without serious injury, or even fatal effects in the case of sensitive roots. When the platinum electrodes were on opposite sides of the apex, the curvature was always towards the positive electrode. If, however, one electrode was placed on the non-irritable base of the root and the other to one side of the apex, the curvature always took place towards the current side, independently of which electrode was on the apex. These results were obtained upon a klinostat into which the current was led by mercury contacts, and transmitted by platinum electrodes to the stimulated region of the root. The seedling and wires within the rotating glass cylinder were insulated on a slab of paraffin wax.

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[*] 'Bot. Zeit.,' 1882, p. 257.

[†] Müller-Hettlingen, 'Pflüger's Archiv,' vol. 31, 1883, p. 193.

[‡] Brunchhorst, 'Ber. d. D. Bot. Ges.,' 1884, vol. 2, p. 204.

[§] Full details will be given by Miss Bayliss in a later paper.

The facts observed suggested that the curvatures were not the result of any parallelo-galvanotropic irritability, but were due to the accumulation of the products of electrolysis at the points of application of the electrodes. Confirmation was obtained by exposing the roots to strong currents (voltage 1 to 4) for short periods (five to eight minutes), and then rotating on a klinostat, when exactly similar results to the above were given. Furthermore, if the anodal region was cut out of an electrolysed root and applied to one side of the apex of another, a curvature was shown to this side. In addition, the application of minute squares of absorbent paper, moistened with decinormal acid or alkali, caused curvatures towards the stimulated side, whereas ordinary neutral paper produced no effect in air saturated with moisture. When the acid and alkali were applied simultaneously on opposite sides, the curvature always took place towards the acid side. This corresponds to the curvature towards the positive (acid) electrode produced by moderately strong currents. The weakest currents used produced similar positive curvatures, and hence Brunchhorst's negative curvatures cannot be explained by Weber's law, as being due to the normal acidity of the root tissues preventing the stronger stimulating action of the acid coming fully into play until it accumulates beyond a certain limit.

The curvatures are usually completed in from 6 to 24 hours after exposure to the current, but they may be distinctly perceptible within four to six hours, and may begin in one to two hours, under optimal conditions. Hence it is not surprising that if the roots are fixed in a plaster cast after stimulation, and rotated on a klinostat for one or two days, a rapid sharp curvature is produced on freeing the root from the cast, whereas after two to four days the effect of the stimulation has passed away. All of these curvatures can be produced without any of the cells of the root being killed, and even when an injury is produced, the curvature is usually towards the injured side, instead of away from it, as in a true traumatropic curvature.

The curvatures produced by continuous currents appear usually to be accompanied or preceded by a temporary more or less pronounced retardation of the average rate of growth in length. Indeed the latter may be temporarily arrested for some time after strong stimulation, even when the electric current produces little or no injury. In such cases negative results may be obtained as regards curvature.

Finally, using non-polarizable electrodes moistened with cell-sap diluted with distilled water, no curvatures were produced, whereas similar stimulation, using platinum electrodes applied to the surface of the root, and with the non-polarizable electrodes still in the circuit so that the resistance was the same, gave the usual curvatures according to how and where the electrodes were applied. With stronger currents and more prolonged exposure, curvatures are induced, even when "non-polarizable" electrodes are used, since the products of electrolysis may diffuse to the surface of the root, and it is impossible to prevent the internal polarization which takes place wherever the current traverses dissimilar saline solutions separated by semi-impermeable membranes. There is, however, less tendency to injury than with platinum electrodes.

The irritable and responsive zone extends 4 to 5 mm. behind the apex of the root of *Vicia Faba* and *Phaseolus vulgaris.* When one platinum electrode was applied to the non-irritable base of a root, and the other laid flat on the extreme tip, no curvature was produced in whichever direction the current was passed. This is presumably due to the products of electrolysis diffusing evenly and stimulating the irritable regions and cells equally on all sides, for when the same current was applied transversely behind the apex, a positive curvature was shown. If the roots were either truly positively or truly negatively parallelogalvanotropic, they should curve in the above experiment so as to place the tip parallel to the current, and either against or with its direction, whenever this does not at first coincide with their tropic irritability.

The "galvanotropism" of roots is therefore due to chemotropic stimulation by the products of electrolysis, of which the acid is more effective than the alkali, the latter also being neutralised more or less by the respiratory carbon dioxide. It is indeed possible that the curvature of the roots of *Lupinus albus* in gelatine towards phosphates and carbonates observed by Lilienfeldt* may be of similar origin, since acid phosphate and alkaline carbonates were used. That the "galvanotropic" or *galvanogenic* curvatures are not traumatropic in origin is shown by the fact that they may be produced without any cells being killed. In Brunchhorst's experiments the electrolysis presumably occurred in the superficial cells of the roots submerged in water, the tissues being sufficiently impermeable superficially to the liberated acid and alkaline ions to allow them to accumulate beyond the minimum for stimulation. Although the curvature is usually sharp and strongly localised to the point of application of the electrode, the discriminatory power of the root, as well as the relation of the rates of growth on concave and convex sides to the normal rate of growth, suffice to show that the response is a stimulatory one, and is not due to the direct action of the products of electrolysis, retarding growth on one side or accelerating it on the other.

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* Lilienfeldt, 'Ber. d. D. Bot. Ges.,' 1905, vol. 23, p. 91.

# *On the Isolation of the Infecting Organism ("Zoochlorella") of* Convoluta roscoffensis.

By Frederick Keeble, M.A., University College, Reading, and F. W. Gamble, D.Sc., University of Manchester.

(Communicated by Sydney J. Hickson, F.R.S. Received October 6, 1905.)

The present paper gives a preliminary account, (1) of experiments proving that the green cells ("zoochlorellæ") of *Convoluta roscoffensis* result from infection from without: (2) of the means whereby the infecting organism may be cultivated outside the body of the animal: and (3) of the nature of the infecting organism.

1. *Evidence for Infection.*—In our former papers* we reached the conclusion that though direct proof of infection was lacking, the evidence pointed most strongly to infection as the source of the green cells of *Convoluta.* We showed, moreover, that the difficulty in the way of obtaining direct proof of the origin of these green cells is due to the fact that the mucilaginous capsules that invest the clutches of eggs laid by *Convoluta* are rarely, if ever, sterile. Even when adults are washed repeatedly in sterilised sea-water and caused to lay in sterilised surroundings, their egg-capsules become covered in time with a varied flora of colourless and of green organisms.

It is therefore necessary to isolate the young at the moment of hatching. During the present summer we have done this in larger numbers than before and maintained them in carefully filtered sea-water. Such young *Convoluta* remain colourless and may be kept in this condition for at least a month without showing any sign of infection, whilst at any time batches of them may be caused to become green in one to three days by the addition of sea-water or of cultures of the infecting organism.

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* "The Bionomics of *Convoluta roscoffensis*," 'Roy. Soc. Proc.,' vol. 72, p. 93, and 'Quart. Journ. Micro. Sci.,' vol. 47, p. 363, 1903.

2. *The Cultivation of the Infecting Organism.*—All attempts to cultivate green cells taken from the body of *Convoluta* have failed. Haberlandt made an unsuccessful attempt, we ourselves were equally unsuccessful, and so also was Miss Harriette Chick, who brought to the task great experience of such researches and the most recent methods.

The problem had therefore to be attacked from the other end. If the green prisoners of *Convoluta* never escape alive, the only chance of obtaining the infecting organism lies in catching it before its entrance into the animal. A scrutiny of many attempts to obtain colourless *Convoluta* in large quantities revealed the fact that generally egg-capsules, isolated in sterile water, give rise to *Convoluta* which remain colourless for a fairly well-marked period of two or three weeks. After this time, however, green specimens make their appearance. Sometimes the number of green animals thus appearing is few; more often it increases with great rapidity. Such results suggested that the infecting organism occurs sporadically on or in the capsules; that it divides freely in this situation; and that after a period of vegetative division it is liberated in sufficiently large numbers to infect the hundreds of *Convoluta* experimented upon.

During the past summer this hypothesis has been put to the test and found to be correct. Large numbers of egg-capsules were kept in filtered water and the young *Convoluta* upon hatching were removed, so that the vessels contained only empty capsules or capsules the eggs of which had failed to hatch. These vessels were kept under observation. At the end of three weeks several minute spherical bodies of a spinach-green colour were detected. Upon microscopical examination these proved to be colonies of green cells enclosed by and filling an egg-capsule. During examination the membrane around such a colony bursts, and the contents, previously quiescent, swarm out of the capsule, revealing themselves as so many unicellular flagellated green organisms.

It remained to apply the infection test. Samples of colourless *Convoluta* reared in sterilised surroundings were put into the vessel containing these flagellated cells. They became infected, and in the course of two or three days exhibited in their tissues green cells identical in character with those of normal *Convoluta roscoffensis.*

Similarly, sterilised sea-water containing cultures of these green organisms is as potent as ordinary unsterilised sea-water in producing infection.

Ordinary sea-water or such cultures alike induce infection within a few days, when added to just hatched *Convolutas* reared previously under sterile conditions; whereas the addition of sterile sea-water to samples from the same stock of *Convoluta* produces no infection.

3. *Nature of the Infecting Organism.*—Whilst reserving for a detailed and illustrated account the full description of the organism infecting *Convoluta roscoffensis,* we may here briefly refer to its more salient characters and systematic position. In its *adult* and *holophytic* stage, the motile green organism is ovoid and flattened in front. At the anterior end it possesses two pairs of similar flagella. A basin-shaped chloroplast envelops the greater part of the body, and is turned in anteriorly, forming a clear border to a colourless funnel-shaped area which runs axially for a third of the cell's length. A plate-like red "eye-spot" or stigma, with a darker rim, lies somewhat excentrically a little in front of the middle of the cell and not in relation to the flagella. The resting nucleus is spherical. Near the posterior end of the chloroplast is a large octagonal pyrenoid, provided with a starch sheath, giving it a somewhat irregular outline. True starch, giving a marked blue colour with iodine, is present in quantity. A cell-wall is either absent or of extreme tenuity in the organism when first liberated, but later on a marked wall of mucilaginous character is demonstrable and may reach a considerable thickness.

These characters indicate that the green cells of *Convoluta roscoffensis* are true algæ, belonging to the Chlorophyceæ and allied to *Chlamydomonas.* The presence of four equal flagella suggests that they belong to the genus *Carteria.**

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* Blackman and Tansley, 'New Phytologist,' vol. 1, p. 23, 1902.

# *Further Observations on the Germination of the Seeds of the Castor Oil Plant* (Ricinus communis).

By J. Reynolds Green, Sc.D., F.R.S., Professor of Botany to the Pharmaceutical Society of Great Britain, and Henry Jackson, M.A., Fellow and Tutor of Downing College, Cambridge.*

(Received March 22,—Read May 18, 1905.)

About 15 years ago one of the authors carried out a series of researches on the germination of the seeds of the castor oil plant (*Ricinus communis*),† and endeavoured to ascertain the course of the decomposition and utilisation of the reserve materials which are present in the seed. As the results of this investigation formed the starting point of the present series of researches, it will be well at the outset to restate the conclusions which were then arrived at.

The larger part of the reserve materials of the seeds of Ricinus, which are laid up in the cells of the endosperm, consists of the well-known castor oil. The amount varies in different seeds, but it ranges from as little as 50 per cent. to upwards of 80 per cent. There is a considerable amount of proteid matter in the cells, most of which is found in the so-called aleurone grains. These have a somewhat intricate structure; an ovoid mass of phytoglobulin, soluble in 10 per cent. solution of common salt, surrounds a proteid crystal, soluble in saturated solution of the same salt. In the grain by the side of the crystal there is a rounded aggregation of mineral matter, the so-called globoid, long considered to be a double phosphate of calcium and magnesium, but probably a more complex body containing its phosphorus in some form of organic combination. According to Vines ‡ the proteids of the grain are an albumose and a globulin; in the opinion of Osborne and Harris § this is not the case, only a globulin being present, probably identical with the *edestin* of the hemp seed. There are other substances present in small amount, but in very trifling proportions when compared with the oil and the proteids.

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* The present series of experiments was commenced by me in collaboration with Mr. W. T. N. Spivey, of Trinity College, Cambridge. After his lamented death in 1901, Mr. Jackson took his place.—J. R. G.

† Green, 'Roy. Soc. Proc.,' vol. 48 (1890), p. 370.

‡ Vines, "Proteid Substances in Seeds," 'Journ. of Physiol.,' vol. 3 (1880), p. 91.

§ Osborne and Harris, "Nitrogen in Protein Bodies," 'Amer. Chem. Journ.,' vol. 25 (1903), p. 335.

The conclusions arrived at in 1888 with reference to the changes set up during germination were the following :—

"1. The reserve materials in the endosperm of *Ricinus communis* consist chiefly of oil and proteid matters, the latter being a mixture of globulin and albumose.

"2. The changes during germination are partly due to enzyme action, there being three enzymes present in the germinating seed: one is a protease resembling trypsin, the second splits the oil into fatty acid and glycerine, the third is a rennet enzyme.

"3. At least two of these, and therefore presumably all of them, are in a zymogen condition in the resting seed, and become active in consequence of the metabolic activity set up in the cells by the conditions leading to germination, especially moisture and warmth.

"4. The changes caused by the enzymes are followed by others, due to the metabolism of the cells, these being processes of oxidation.

"5. The embryo exercises some influence on the latter, setting up as it develops a stimulus probably of a physiological description.

"6. The result of these various processes is to bring about the following decompositions :—

> "The proteids are by the enzyme converted into peptone, and later into asparagin.
>
> "The oil is split by the glyceride enzyme into fatty acid and glycerine; the latter gives rise to sugar, and the former to a vegetable acid which is soluble in water and in ether, is crystalline, and has the power of dialysis.

"7. Absorption in all cases takes place by dialysis.

"8. The appearance of starch and of oil in the embryo or the young plant is due to a secondary formation, and not to a translocation of either."

### FORMATION OF LECITHIN.

The advances in our knowledge of the metabolic processes of plants that have been made during the interval that has elapsed since the publication of this paper, and the new methods of experiment that have been introduced, suggested that the work which was admittedly incomplete and tentative should be taken up again. There remained especially the question of the meaning of the reserve supplies of phosphorus and the part which they take in the general metabolism accompanying germination. The aggregates of phosphates referred to as the globoids of the aleurone grain undergo a change during the process, by virtue of which they slowly pass into solution.

As this change supervenes upon the development of an acid reaction in the seeds, it seems not unlikely that it may be caused by the action of the organic acid which is formed in the cells of the endosperm almost as soon as germination begins.

On resuming the work a more careful examination of the oily contents of the endosperm cells led to the discovery that they contained, mixed with the oil, a certain quantity of a substance, the decomposition products of which pointed to its being a lecithin (a peculiar fatty body containing phosphorus). The resting seeds were pounded in a mortar till they formed a homogeneous paste. This was extracted for some hours with ether in a Soxhlet's apparatus, and was afterwards twice extracted further on a water-bath with absolute alcohol, the flask being fitted with a reflux condenser. The alcoholic and ethereal extracts were mixed and evaporated to dryness at a gentle heat on a water-bath, and the fatty residue fused with dry carbonate and nitrate of potassium until all trace of free carbon had disappeared. After cooling, the fused residue was dissolved in water, and the addition of ammonium molybdate and nitric acid produced a yellow precipitate, indicating the presence of phosphorus. The quantity of the latter was ascertained by converting it into magnesium pyrophosphate and weighing.

A little of the oil was then hydrolysed by boiling with baryta, when there separated out a flocculent precipitate of a barium salt, which, after washing and drying, was found to contain no phosphorus. This barium salt had the characteristic soapy appearance of the stearates. It was found possible to identify cholin in the endosperm of the germinating seeds, as will be more fully shown a little later (p. 74).

The only way of estimating the amount of the lecithin present in the alcohol-ether extracts of the endosperm was to determine accurately the phosphorus as magnesium pyrophosphate. Great precautions were taken to ensure the repeated use of very dry ether so as to exclude the possibility of extracting any inorganic phosphates. A little of the oily residue from the extraction was incinerated in a platinum dish, and it was found to leave no inorganic ash. We therefore assumed that all the phosphorus extracted as described was originally present in the complex lecithin form. Taking the formula usually given for lecithin ($C_{44}H_{90}NPO_4$), we calculated the amount of the latter that would be present. Its average amount was equal to 0·236 per cent. of the dry weight of the seeds.

Several series of experiments were made to investigate the changes in the fatty constituents of the endosperm during germination. The action of the fat-splitting enzyme known now as *lipase* was confirmed, and the early stages of germination were found to be as set out in the former paper. Consequently, examinations of the contents of the seeds were made at certain stages of the germination, and before the process had begun. The stages were the following:—(1) The seed at the time of the cracking of the testa, usually after 24 to 48 hours in the soil; (2) the seed with the radicle protruding for a length of 1 to 2 cm., usually about three days after sowing; and finally, (3) seeds whose lateral root system had become fairly well developed. The times at which these stages were reached varied with the samples of seeds used, and the temperatures at which the germination took place.

The results of a typical experiment are stated in the subjoined table:—

Table A.

| Degree of development. | Dry weight of seeds used. | Oil in seeds. | | Fatty acid in seeds. | | Lecithin per cent. of weight of seeds. |
|---|---|---|---|---|---|---|
| | | Actual weight. | Percentage of weight of seeds. | Actual weight. | Percentage of weight of seeds. | |
| | grammes. | grammes. | | grammes. | | |
| Resting seeds ............ | 4·48 | 3·7115 | 82·8 | 0·1 | 2·2 | 0·236 |
| Seeds just cracking testa | 4·47 | 3·016 | 67·5 | 0·204 | 4·6 | 0·17 |
| Radicle protruding 1—2 cm. | 4·17 | 2·19 | 52·5 | 0·5 | 11·9 | 0·475 |
| Lateral roots spreading. Root system established | 3·34 | 0·789 | 23·6 | 0·565 | 16·89 | 0·873 |

It will be seen that the amount of lecithin diminished during the early stages of germination, the reserve supply becoming almost exhausted. After the young seedling had begun to develop, however, there was a gradual increase in the amount. This increase was maintained during the later stages and was fairly constant till the endosperm was used up. There was clearly a consumption of the oil throughout.

The amount of lecithin, though small, varied somewhat in different experiments. In one series it was in much larger proportion than in that quoted. The residue soluble in alcohol and ether amounted to 0·9 per cent. of the weight of the resting seeds, and in the later stages of germination the amount present rose to approximately 2 per cent. This quantity, however, in our experiments was exceptional.

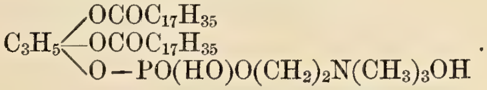
These experiments suggest that in the utilisation of the fatty reserves lecithin certainly plays a part and, possibly, a predominant part.

Lecithin has been shown by Overton * to be a normal constituent of living cells, and to exercise considerable influence on the transport of various materials across the limiting layers of the protoplasm. It has no doubt also a certain, though at present undetermined, nutritive value.

The composition of lecithin is indicated by the change which it undergoes on hydrolysis, when it is decomposed into *stearic* (or *palmitic* or *oleic*) *acid, glycero-phosphoric acid* and *cholin.*

$$\underset{\text{Lecithin.}}{C_{44}H_{90}NPO_9} + 3H_2O = \underset{\text{Stearic acid.}}{2C_{18}H_{36}O_2} + \underset{\substack{\text{Glycero-}\\ \text{phosphoric}\\ \text{acid.}}}{C_3H_9PO_6} + \underset{\text{Cholin.}}{C_5H_{15}NO_2}.$$

From this, its constitution has been represented as



$$C_3H_5\begin{cases} OCOC_{17}H_{35} \\ OCOC_{17}H_{35} \\ O-PO(HO)O(CH_2)_2N(CH_3)_3OH \end{cases}.$$

Only a trace of it exists in the resting seed; as it increases during germination and the quantity remains fairly constant during the whole period of absorption of the fatty reserves by the seedling, we have evidence of a formation of it during the germinative processes. The endosperm contains such substances as may yield the several groups necessary for its formation. The decomposition of the oil by the enzyme *lipase* can furnish the fatty component, belonging to the oleic group, and at the same time the glycerine of the glycero-phosphoric acid. The phosphorus of the latter is at hand in the shape of the phosphatic globoids whose solution has already been alluded to. The nitrogenous body cholin may be looked for among the products of the decomposition of the proteids of the aleurone grains.

Examination of the contents of the endosperms during germination ultimately established the presence of all these constituents. The fatty acids and the glycerine were identified in 1888, and the methods of detection and estimation were quoted in the former paper. A careful examination of the phosphates of the globoids, taken for purposes of comparison from seeds at the respective stages of germination quoted in Table A (p. 72) showed that their solution proceeded side by side with that of the oil.

No change in them could be observed under the microscope till the testa was cracking, and the time of its inception varied a good deal. In the early stages, prior to such cracking, no reaction for phosphorus could be obtained from a watery extract. The quantity of phosphorus present in the resting seed was 0·205 per cent. of the dry weight; this diminished in Stages 2, 3, and 4 of Table A to 0·16, 0·14, and 0·11 per cent. The globoids are decomposed gradually but fairly rapidly during the germination, and in the later stages contribute to the acidity of the cell-sap, which contains phosphoric acid.

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* Overton, 'Pringsheim's Jahrb.,' vol. 39 (1900).

Search was made in a mass of endosperms for cholin. The germinating seeds were ground up in a mortar and allowed to stand for some days under alcohol which was nearly absolute. This was decanted and evaporated to dryness, the residue being again extracted with absolute alcohol and subsequently by a mixture of alcohol and ether. These extracts were mixed and evaporated to dryness, leaving a final residue, soluble in water. When a strong aqueous solution of this was boiled, decomposition took place, and a gas was evolved which possessed the well-known ammoniacal and fishy odour characteristic of tri-methylamine. The decomposition can be represented by the following equation:—

$$CH_2(OH)CH_2N(CH_3)_3OH = CH_2(OH)CH_2OH + N(CH_3)_3.$$

When to some of an aqueous solution of the residue from the alcoholic and ethereal extracts a little platinic chloride was added, after standing for some time the characteristic yellow octahedral crystals of the compound which cholin forms with platinic chloride separated out. These were soluble in 15 per cent. alcohol, and on combustion yielded a residue of metallic platinum. We have thus all the constituents of lecithin present in the germinating seeds.

It was difficult to apply the ordinary tests for lecithin when so large a quantity of oil was present. Towards the close of the germination, however, conditions were more favourable, the lecithin being present in relatively large proportion.

The existence of a proteolytic enzyme of a tryptic nature in the germinating seeds was shown in the former paper. Among the products of its action a considerable quantity of crystalline amino-bodies were detected, though not sufficient for a complete analysis. They separated out from the concentrated alcoholic extracts, after removal of the sugars, in quantities that enabled their amino-nature to be proved. The power of the enzyme to produce these *in vitro* has already been noted.* We have found the cholin also to be due to the action of this enzyme. 150 cc. of an extract of the endosperms of a quantity of germinating seeds was prepared by steeping them for several hours in water containing 0·2 per cent. of formaldehyde as an antiseptic. It was then strained through muslin and filtered till it appeared as a clear straw-coloured liquid. This was divided into two, and half of it boiled to destroy the protease. A quantity of globulin was prepared from a further quantity of the same germinating seeds by extracting them with a 10 per cent. solution of common salt, and precipitating the proteid by addition of alcohol. The precipitate was rapidly collected on a filter, washed and suspended in a little water.

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* Green, *loc. cit.*, p. 377.

The 75 c.c. of the extract that had not been boiled was put into a beaker and 5 c.c. of the suspended globulin added; a similar preparation was made of the 75 c.c. that had been boiled. Both were kept in an incubator at 40° C. for a week. At the end of that time digestion was complete in the unboiled preparation, the globulin having disappeared, leaving a morbid solution. Both were perfectly free from bacteria, the formaldehyde acting extremely efficiently as an antiseptic. The two digestions were then filtered and the filtrates evaporated to dryness. The residues were extracted successively with absolute alcohol and with a mixture of absolute alcohol and ether, each extraction being continued for two days. The first alcoholic extract was evaporated to dryness and the residue again extracted with ether. The two ethereal extracts were subsequently mixed and evaporated to dryness and the residue taken up with a little water. There was considerably more of this residue in the digestion carried out by the unboiled extract of the seeds than in that associated with the other. To each a little platinic chloride was added in watch-glasses, and they were set aside. After 24 hours, in both cases minute crystals had settled to the bottom of the liquid, which were soluble in alcohol of 15 per cent. concentration. From this solution the characteristic yellow octahedra slowly settled out, and these gave the same reactions as those prepared from the extracts of the endosperms. The amount obtained from the digestion by the unboiled extract was much greater than that from the boiled one, though the latter yielded some, attributable no doubt to a certain quantity present in the 75 c.c. of the original extract of the seeds employed.

The experiment shows, therefore, that the cholin of the lecithin can be prepared from the proteids of the seeds by an enzyme which is developed during germination, and is presumably the enzyme already described as a trypsin.

The similarity of this enzyme to the trypsin of the pancreas is borne out by the occurrence of tryptophane among the products of its activity both in the plant and *in vitro* in the laboratory.

The contribution of material for the synthesis of lecithin does not seem, however, to be the only result of the decomposition of the fat. There is not sufficient phosphorus in the resting seeds to enter into the composition of as much lecithin as the fat would produce. It is, of course, possible that the lecithin may be decomposed during consumption and part of its phosphorus set free to combine again, but even then the quantities do not seem to be proportional. Another fate must attend a considerable quantity of the fat. To this point we shall return later.

These results suggest that the utilisation of the oily reserves is a much more complicated process than was supposed. The enquiry took from this point a wider range, and soon involved the abandonment of the idea that the separate reserves undergo independent changes during germination.

## THE SUGARS OF RICINUS.

A more complete study of the sugar was next undertaken. Du Sablon showed, in 1895* that it is a mixture of at least two sugars, one of which has not the power of reducing Fehling's solution. In our experiments, a large number of seeds having been germinated, the endosperms were separated from the embryos and ground to a paste in a mortar. The mass was then extracted with large quantities of water, by keeping it for some hours in a steriliser at 100° C., removing the water at intervals till the extract showed that all the sugar had been dissolved. The extracts were mixed and concentrated to about one-tenth of their volume. Addition of normal acetate of lead separated from this extract the acids present, together with the bulk of the proteids and certain other constituents. These were filtered off, and the sugars were precipitated from the filtrate by adding basic lead acetate and ammonia. The precipitate was separated by filtration and suspended in water, and the lead removed by a stream of sulphuretted hydrogen. The solution so obtained was concentrated, and the process repeated, the final solution being then concentrated to a thick syrup, which showed the presence of two constituents possessing different solubilities in alcohol. By a repetition of concentration and extraction, the syrup was ultimately separated into two parts, one of which reduced Fehling's solution, while the other did not. Unfortunately the separation did not involve the complete isolation of the two sugars, as the reducing power of the first fraction was always increased after boiling with dilute mineral acid. The increase was not constant in different preparations, a fact which pointed to incomplete separation rather than to the reducing sugar being of the maltose type.

The second fraction of the syrup was, however, free from the reducing sugar. Treated with invertase or with a dilute mineral acid it speedily reduced Fehling's fluid. A quantity of it was concentrated nearly to dryness and with some difficulty dissolved in alcohol. Addition of ether to a little of the solution caused precipitation of the sugar. To the great bulk of the solution, therefore, a little ether was added, drop by drop, till a faint turbidity was apparent.

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* Du Sablon, "Sur la Germination des Graines Oléagineuses," 'Rev. Gén. de Bot.,' 1895, p. 145.

After standing in this condition for some days, a crop of aggregates of crystals separated out. When dissolved in water they were found to have a specific rotatory power of about $\alpha_D = +66$. After inversion with a dilute mineral acid the specific rotatory power became about $\alpha_D = -18$. The solution of the crystals gave no crystalline osazone on warming with phenylhydrazine acetate.

These reactions are fairly conclusive that the non-reducing sugar is cane-sugar.

The reducing sugar was refractory and no method succeeded in rendering it crystalline. It was also found impossible to separate it completely from the cane-sugar, so that its specific reducing power could not be obtained. Readings with the polarimeter were unsatisfactory on account of its proving impossible to free its solutions from a yellow colouration. When the latter were warmed with phenylhydrazine acetate they yielded a quantity of a pale yellow osazone which analysis proved to be the osazone of a hexose. After several recrystallisations from alcohol and from ethyl acetate the crystals were found to have a constant melting point at 204° C. This is consistent with the view that it is invert sugar produced from the cane-sugar with which it is associated. It negatives the hypothesis put forward in the former paper that it is derived from the glycerine of the fat, for this sugar (glycerose), now much more completely investigated, is known to yield an osazone melting at 130° C. to 131° C.*

The occurrence of two sugars exhibiting the characters just described suggested a search for invertase among the constituents of the endosperm. A good number of well germinated seeds were selected, having most of the endosperm absorbed; the embryos were well developed, their root system considerably branched. The endosperms were removed and ground up into a paste, which when strained through muslin yielded 95 c.c. of an acid sap. This was carefully neutralised and a little antiseptic added. It contained a quantity of both reducing and non-reducing sugar, 10 c.c. of the sap reducing 0·2 gramme of cupric oxide. Tubes were prepared containing respectively 10 c.c. of the neutralised juice with 10 c.c. of a solution of the non-reducing sugar from the seeds, and 10 c.c. boiled juice with the same quantity of the sugar solution, and they were digested in a water-bath at 40° C. for several hours. On titration the weight of cupric oxide reduced by the digestion containing unboiled juice was 0·31 gramme while the other gave the same weight as the original juice, 0·2 gramme. The treatment with the juice had increased the original reducing power 50 per cent., showing the presence of invertase.

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* Fischer and Tafel, 'Ber. d. deut. Chem. Ges.,' vol. 20, p. 1088; Fenton and Jackson, 'Trans. Chem. Soc.,' 1899.

Further experiments upon the same point showed that invertase appears in the endosperms at a very early period of germination, usually after a few hours; it is well established in 48 hours, and increases in amount up to the stage at which a good root system has been established. In a series of experiments upon its development during the germination three stages were compared: (1) The seeds had the radicle protruding about 0·3 inch; (2) The roots were 1 inch long and the secondary rootlets were just cracking the primary root; (3) There was a good root system and the endosperms were about half consumed. Extracts were made of all these and 2 c.c. of each allowed to act on 20 c.c. of a 1 per cent. solution of cane-sugar, at 40° C. for 24 hours. They were then titrated with Fehling's fluid, when the weights of cupric oxide obtained were:—

(1) 0·003 gramme; (2) 0·006 gramme; (3) 0·007 gramme.

These experiments lead us to the conclusion that the sugars of the endosperms may be put down as cane-sugar and invert sugar.

The relative quantities of these two sugars during the progress of germination have been ascertained and are given in Table B. Experiments on this point have been published by Du Sablon in the paper already referred to. He states that he found non-reducing sugar to be slightly in excess of reducing sugar in the resting seed and to increase more rapidly than the latter till the radicle is about 1·5 to 2 inches long, when the reducing sugar becomes equal in amount and, later on, preponderates considerably.

Our experiments were carried out in the following manner:—A number of seeds were germinated in sawdust in an incubator kept at a temperature of 22° C. In each experiment three were taken, peeled, and ground up to a smooth paste in an agate mortar. The paste was then boiled with a sufficient quantity of water for an hour, the extract strained off, filtered, and divided into two. Half was warmed to 40° C. with 1 c.c. of a solution of invertase prepared from yeast, and kept at that temperature for 24 hours. The invertase solution was ascertained to be free from sugar or other substance capable of reducing Fehling's fluid. The two halves of the extract were then titrated side by side, and the weight of the cupric oxide taken in each case. From these weights the quantities of the two sugars were computed in the usual way.

Table B.

| Time of germination in hours. | Condition of seeds when ground up. | Invert sugar in milligrammes. | Cane-sugar in milligrammes. |
|---|---|---|---|
| 0 | Resting seeds | 1·1 | 10·7 |
| 45 | Caruncle swollen | 2·7 | 5·17 |
| 69 | Little further external change | 2·3 | 0 |
| 117 | Root about 0·75 inch long | 6·7 | 19·4 |
| 168 | Root 1·5 inch long | 5·2 | 10·5 |
| 216 | Roots branching | 19·5 | 35·7 |
| 240 | Endosperms cracking | 29·01 | 35·8 |
| 312 | Good root system | 40·8 | 52·6 |

A comparison of this Table with Table A suggests that the course of events in which the sugars are involved proceeds upon much the same lines as that connected with the lecithin. The cane-sugar is present in greater quantity in the resting seeds, it gives place to invert sugar under the influence of the invertase during the early period of germination, and subsequently increases in amount and remains slightly in excess of the invert sugar during the later stages when absorption is more active. This suggests that cane-sugar is the actual reserve, and that the invert sugar represents the form which has the greater nutritive value.

In accounting for the increase in the quantity of cane-sugar which marks the progress of germination, it is necessary to call attention to a fact noticed for the first time a few years ago by Mr. Biffen in the Cambridge Botanical Laboratory. Emphasis has already been laid upon the fact that a very vigorous metabolism in the endosperm cells is an accompaniment of germination. This was commented on by Van Tieghem* in 1877, when he found that endosperms deprived of their embryos were capable of swelling and apparently starting a kind of development. In the former paper on this subject one of us described experiments† confirmatory of Van Tieghem's views. Biffen has found that a considerable increase of the protoplasm of these endosperm cells is a marked feature of the early stages of germination. The exact time at which it occurs varies somewhat, but it corresponds fairly closely with the recommencing formation of cane-sugar. The coincident occurrence of these two events points to a growth of the protoplasm of the endosperm cells at the expense of the initial reserves, which we have seen are undergoing conversion changes at and before this time, and a subsequent construction of further carbohydrate reserves by

* Van Tieghem, "Sur la Digestion d'Albumen," 'Comptes Rendus,' vol. 84, p. 578.

† Green, *loc. cit.*, p. 389.

the protoplasm in the endosperm for the nutrition of the outlying embryo as its growth continues. Apart from such secretion the endosperm contains no carbohydrate material, while the latter seems to be essential for the maintenance of merismatic tissue. The fact that this carbohydrate substance is cane-sugar coincides with the observation of Brown and Morris* that cane-sugar is always present in the growing embryo of the barley-grain. It appears to be a form of carbohydrate very suitable for serving as a temporary reserve material, more easily utilisable than starch, and therefore formed where the deposit of the reserve will be of very short duration, as in the case of the embryo, and in that of the foliage-leaf, where Brown and Morris found it at a very early period of the photosynthetic construction. Indeed, from the results of analyses of the mixed sugars then present they suggested that it might even be the first sugar formed.†

It may again be noted that in the case of Ricinus its formation is accompanied or speedily followed by the secretion of invertase. The enzyme is not present in the resting seeds, but develops in the endosperms after exposure to a temperature of 25° C. in moist earth or sawdust for 48 hours or less, though germinative changes are not visible so soon in the external appearance of the seeds. The amount of the enzyme increases continuously all the time of germination, and the invert sugar increases coincidently. The protoplasm appears to keep up a secretion of cane-sugar and the invertase seems to keep working on the latter, so as to supply invert sugar at once to the protoplasm of the cells and to the young absorbing embryo.

It will be seen from what has been said that we do not associate the formation of this carbohydrate material during the germination *directly* with the diminution in quantity of the oil which is taking place at the same time. Our experiments lend no support to the views of Sachs that the oil was directly transformed with either sugar or starch. The two processes are features of a new metabolism set up in the cells as germination becomes established. To this point we shall return later.

## THE ACIDS OF THE GERMINATING SEEDS.

The question of the nature of the acid to which the reaction of the germinating seed is due remains to be dealt with. Evidence of acidity can be obtained after a seed has been exposed to warmth and moisture for 24 hours, and it becomes more and more intense for six or seven days.

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* Brown and Morris, "Researches on the Germination of some of the Gramineæ," 'Journ. Chem. Soc.,' vol. 57 (1890), p. 518.

† Brown and Morris, "A Contribution to the Chemistry and Physiology of Foliage Leaves," 'Journ. Chem. Soc.,' May, 1893, p. 673.

While the reaction to litmus paper becomes very prominent, only very small quantities of acid can be obtained from the seed. The expressed juice of a parcel of germinating seeds was titrated with decinormal potash solution, and 10 c.c. of it neutralised only 4 c.c. of the alkaline solution. We made several attempts to prepare it in quantity by experimenting upon about a thousand seeds at once. They were germinated for a week, and the endosperms separated from the embryos, ground and boiled in water in a steriliser for several hours. After straining and filtering part of the extract was distilled by the aid of steam. The distillate was practically neutral in reaction, the merest trace of acidity coming over. The acid in the remainder, after removal of uncoagulable proteid, was precipitated by normal lead acetate, and the lead salt filtered off, suspended in water and treated with a stream of sulphuretted hydrogen till the lead was all converted into sulphide. The filtrate from the latter was concentrated to a small bulk, and the precipitation and subsequent treatment repeated. The final filtrate was concentrated to a small bulk *in vacuo* over sulphuric acid.

The acid residue, somewhat syrupy in consistence, was then washed repeatedly with dry ether, which dissolved a certain quantity, leaving behind, however, a good deal of acid which was soluble in water only. The bulk of the latter was ascertained to be phosphoric acid. The solution in ether was concentrated *in vacuo* and formed a syrupy residue. We found it impossible to crystallise this acid or to obtain a crystallisable salt. Many attempts were made to effect crystallisation, but in only one case was any success obtained, and then only a few crystals on the surface of the syrup were formed. Unfortunately, therefore, the nature of the acid has not been ascertained.

After looking for the source of this organic acid we again find reason to attribute it to the oil. We have already pointed out (p. 75) that the amount of lecithin formed is not sufficient to account for the disappearance of the whole of the oil of the seed, but that another fate awaits a considerable quantity. It was suggested in the former paper* that the acid of the germinating seed was derived from the oil by certain processes of oxidation, and served as the means of its utilisation. It is extremely unlikely that this acid is directly or indirectly connected with the sugars. We think we have here the explanation of the gradual diminution of the oil in the early stages of germination, and of the development of the coincident acidity. The acid reaction of the endosperm sets in before any change can be detected in the globoids of the aleurone grains and before any reaction for phosphoric acid is obtainable. The probability of an oxidation of the oil taking place in the early stages of germination has already been pointed out. This is now rendered still more probable by the discovery of an oxidase in the germinating seeds. On mixing a strained and filtered extract of the endosperms with a solution of hydroquinone, the colour of the latter speedily becomes pink and, later, red. The extract gives instantaneously a blue colour with an emulsion of guaiacum, and slowly turns a solution of pyrogallol purple. Boiling the extract destroys the power of setting up these changes. The oxidase adheres very tenaciously to the tissue of the endosperm, and it is very difficult to extract it completely.

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* Green, *loc. cit.*, p. 385.

Though the oxidase can be extracted and the extract found to act on such easily oxidisable bodies as those mentioned, no attempt has succeeded in making it oxidise ricinoleic acid outside the plant. This may, however, be due to non-attainment of the conditions which exist in the cells of the endosperms. Though its appearance is suggestive, it has not been proved that it plays a part in the oxidative processes of the fats, if the latter take place. The probability of such oxidative processes is considerable, for, in addition to the considerations just put forward, it should be remembered that one of us has shown that the formation of the acid is dependent upon the access of oxygen. In seeds germinated in its absence, though part of the oil was transformed, no acid soluble in water was formed.*

The problem is complicated by the fact that the distribution of the lipase, invertase, and oxidase of the germinating seed is practically the same.

### NUTRITION OF THE EMBRYO.

The sequence of changes which has, so far, been described, suggests a modification of the views now current as to the mode of utilisation of reserve materials in albuminous seeds. It has been commonly held that the efforts of the parent plant ceases with the deposition of reserve food in or near the embryo, in such a condition as to be easily used. Possibly, also, certainly in some cases, the parent is responsible for the provision of an enzyme to effect the change of the reserve food into a suitable condition for absorption. The utilisation is, however, attributed more or less fully to the embryo. In many cases the latter secretes the enzymes itself, and in others it is the active agent in absorption. The metabolic changes in the endosperm attributable to the parent are held to be more or less independent of each other, and to consist of the enzyme actions only, each enzyme fitting its appropriate food for absorption.

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* Green, *loc. cit.*, p. 389.

This, as we have shown, is far from being the case with *Ricinus*. Here we have a series of most complex changes set up by the parent in the endosperm, accompanied by a renewed growth and revived secretory activity of the parent itself. The various constituents are made to act upon each other under the influence of the protoplasm of the endosperm cells, the latter showing a great increase in the amount of their protoplasm, while the protoplasm initiates a complex metabolism comparable in intensity with any which can be marked in the adult plant. It feeds itself, having prepared the food from the reserves; it secretes new products, which were represented but sparingly in the original cell-contents, thus preparing a new and completely representative food supply which it places at the disposal of the embryo. At the same time, however, the latter plays a considerable part in the scheme of nutrition, besides carrying out the processes of absorption.

A study of the distribution of the enzymes of the seed shows us that the preparation of food is not all carried out by the parent. The lipase was stated in the earlier paper* to originate in the endosperm cells and to continue to be developed there during the whole course of the germination. The invertase and the oxidase appear to have a distribution similar to that of the lipase. The trypsin, however, originates in the embryo.

In the course of the researches made by Mr. Biffen, which have already been referred to, he found that the epidermis of the young cotyledons contained cells, occurring at short intervals, which stained quite differently from the rest, and were full of granular contents. We prepared a large number of cotyledons from seeds in course of germination, taking them at an early stage when it was just possible to separate them cleanly from the endosperm. They were then washed carefully in warm distilled water till all organic matter was removed from their surfaces. Each cotyledon was then cut in half along the mid-rib. One set of halves was dipped for a moment in boiling water. The two sets were put into a solution of the globulin of the seeds prepared by dissolving it from the seed in 10-per-cent. solution of common salt and precipitating it by strong alcohol. The tubes containing them were put for a few hours into an incubator at 30° C. At the end of this time the uninjured epidermis had produced such a change in the globulin that the solution gave a vivid reaction for tryptophane on addition of a little chlorine water. The contents of the other tube were unchanged. The presence of trypsin in the cotyledonary epidermis was consequently proved. An extract of the cotyledons gave the same results. Taking these experiments in conjunction with Mr. Biffen's observations, there can be little or no doubt that the special cells alluded to secrete the trypsin.

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* A curious misstatement of what I said on this point in my earlier paper has been made by Connstein, Hoyer, and Wartenburg ('Ber. d. d. Chem. Ges.,' vol. 35 (1902), p. 3988), and recently repeated by Vierling ('Journ. Suisse de Chim. et Pharm.,' vol. 42, (1904), p. 391). I am represented as saying that the action of the lipase is stopped by the liberation of the acids in the endosperm. My paper contains no such statement. What I said was that if the enzyme was set to work *in vitro* in the presence of *dilute hydrochloric acid* it was rapidly destroyed. Reference to my paper will show that I regarded the organic acids formed in the endosperm helpful and not deleterious.—J. R. G.

These observations throw a light upon certain phenomena already alluded to, which were first recorded by Van Tieghem,* and subsequently corroborated by one of us.† Van Tieghem dissected the embryos out of seeds of Ricinus and exposed the endosperms on damp moss for some weeks to a temperature of 25 to 30° C. After several days of this exposure he found them growing considerably, and at the end of a month they had doubled their dimensions. The change was caused by the enlargement and partial separation of the constituent cells. In the interior of the cells he found the aleurone grains to be gradually dissolving, and the oily matter to be slowly diminishing. In the confirmatory experiments made by one of us the changes were found to be much more rapid when pieces of the cotyledons were left in contact with the endosperms than when the embryo was entirely removed. No satisfactory explanation of these phenomena was forthcoming at the time that they were observed, but the discovery that the tryptic enzyme is secreted by the cotyledons affords one. That a very slow germination takes place in the complete absence of the cotyledons may be explained by a small exudation of the enzyme from the latter before their removal or by the endosperm-cells themselves secreting a small quantity of it when the growth of the protoplasm is resumed during the early stages. The diffusion of the trypsin from the cotyledons into the tissue of the endosperm is exactly paralleled by the diffusion of diastase from the scutellum of the barley grain, described by Brown and Morris.‡

## CONCLUSIONS.

The germination of the seed of Ricinus is shown by the experiments now recorded to be associated with a remarkable activity of the cells of the endosperm, which spring into renewed life and set up a very complex metabolism. Their protoplasm grows and takes a prominent part in these metabolic changes, secreting enzymes, and setting up various chemical changes in the cells partly by means of the latter and partly independently of them. In this renewed activity the embryo also takes a share by contributing to the enzyme-formation. The result is the production of a great variety of nutritive material, partly the direct product of enzyme-action, partly produced by the secretory activity of the protoplasm and partly by the interaction of the products of the first two agents. Two varieties of sugar, lecithin, fatty acids, and the products of their oxidation, proteids, and the products of their digestion, including various crystalline nitrogenous bodies, amino- and amido-compounds at least are present. In this mass of nutritive material the embryo is plunged, and by the delicate epidermis of its cotyledons it absorbs, probably selectively, what it needs for its own growth. It is not easy to follow the process of absorption in detail, on account of the metabolism accompanying growth, which is very speedily set up in the cells of the embryo.

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* Van Tieghem, "Sur la Digestion d'Albumen," 'Comptes Rendus,' vol. 84 (1877), p. 578.

† *Loc. cit.*, p. 389.

‡ Brown and Morris, "On the Germination of some of the Gramineæ," 'Journ. Chem. Soc.,' vol. 57 (1890), p. 495.

Analyses of the cotyledons show them to contain a varying quantity of lecithin, amounting in some cases to 1·36 per cent. of their dry weight. Both the sugars can be detected in them, the relative amounts, however, varying, but cane-sugar being usually present in largest quantity.

The reaction of the sap is acid, traces of phosphoric acid being mixed with an organic acid whose nature has not been ascertained. In fact, the transport of the nutritive substances to the embryo seems to be much the same in character as their transport in the tissue of the endosperm. Probably in both cases the presence of protoplasmic threads in the various cell-walls plays an important part in the matter; it seems at any rate probable that this agency is necessary to explain the transport of lecithin to the embryo. A very small quantity of lecithin can be dissolved in water or exist as a fine emulsion. It is improbable, however, that it can be transmitted through the cell-walls by dialysis alone. Dialysis no doubt plays a large part in the absorptive processes, especially where the crystalline substances are concerned.

The renewed metabolism in the endosperm-cells thus furnishes a mass of nutritive material on which both the endosperm-cells and the young embryo feed, and there seems to be no particular difference in the manner in which they are severally nourished.

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# *On certain Physical and Chemical Properties of Solutions of Chloroform and other Anæsthetics.—A Contribution to the Chemistry of Anæsthesia. (Second Communication.)*[*]

By Benjamin Moore, M.A., D.Sc., Johnston Professor of Bio-chemistry, University of Liverpool, and Herbert E. Roaf, M.D., Toronto, Johnston Colonial Fellow, University of Liverpool.

(Communicated by Professor C. S. Sherrington, F.R.S. Received October 19,—Read November 23, 1905).

In a previous communication[†] we have shown (1) that chloroform has a much higher solubility in serum or hæmoglobin solutions than in water or saline; (2) that at the same vapour-pressure of the chloroform the amount dissolved in serum or hæmoglobin solution is considerably higher than in saline or water; (3) that the curve of chloroform vapour-pressure and concentration in solution in the case of water and saline is a straight line, while in the case of serum and hæmoglobin solution it is a curve showing association at the higher vapour-pressures; (4) that in the case of serum addition of chloroform causes an opalescence and a slow precipitation at room temperature (15° C.), and at body temperature (40° C.) a rapid, though incomplete, precipitation, also in the case of hæmoglobin, 1·5 to 2 per cent. of chloroform causes a change of colour and commencing precipitation at room temperature, which becomes almost complete in the thermostat at 40° C., while 5 per cent. and over causes complete precipitation, even at 0° C.

In our previous paper we also recorded the relations between chloroform vapour-pressure and concentration of the anæsthetic in solution for water, saline and serum, throughout a range from below the anæsthetising value to nearly saturation, and gave curves illustrating these relationships.

From these experiments we drew the conclusion that chloroform forms an unstable chemical compound or physical aggregation with the proteids experimented upon, and that it is carried in the blood in such a state of combination. Since proteids build up the living protoplasm, the inference was drawn that chloroform and other anæsthetics must form similar unstable compounds with protoplasm, and that anæsthesia is due to the formation of such compounds, which limit the chemical activities of the protoplasm. On account of the instability of the compounds, these remain formed only so long as the pressure of the anæsthetic in the solution is maintained.

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[*] Part of the expenses of the investigations have been defrayed by a grant from the Royal Society (Government Grant).

[†] 'Roy. Soc. Proc.,' vol. 73, 1904, p. 382.

Since the publication of our paper we have extended our observations to certain tissues in the fresh condition, to emulsions in saline of the ethereal extracts of the tissues of equal concentration to the tissues in "lipoid" or ethereal extractives, and to estimations of the relative solubilities in water and serum of a number of other anæsthetics, and observation of the effects of such other anæsthetics upon the proteids of the serum.

The additional experiments show that the proteids of the tissues combine with chloroform in a similar fashion to the proteids of the serum, and also that other anæsthetics possess higher solubilities in serum than in water, and that the effects in the case of each anæsthetic upon the proteids of the serum, as the anæsthetic is increased in concentration, are similar to those already recorded in the case of chloroform, although the points of commencement of opalescence and precipitation vary quantitatively in the different cases.

Experiments have also been made upon the relative variations in depression of the freezing point caused by additions of chloroform to water, saline, and serum respectively, as also of the variations in electrical conductivity produced in saline and serum respectively by additions of chloroform.

The various series of experiments can best be described under the headings indicated above.

### A.—*Experiments on the Relationship between Vapour-pressure and Concentration of Chloroform in certain Tissues in the Fresh Condition.*

The experiments were made in the "differential densimeter" described in our previous paper, and following the procedures and precautions there discussed, and it is accordingly only necessary here to state how the tissues were prepared for introduction into the densimeter. The tissue concerned was cleared as far as possible from blood; in the case of the heart muscle and liver by perfusion of saline through the organ, and in the case of the brain by dissecting out the larger vessels and washing with saline solution. Adherent connective tissue and fat were removed as completely as possible and the tissue was then passed through a machine sold for comminution of tissues for subcutaneous injection. The mass so obtained is too thick in consistency for introduction into the densimeter and was made thinner by addition of an equal volume of normal saline. The tissues were so prepared in as fresh a condition as possible, and the various dilutions of chloroform were made as described in our previous paper. The emulsion of tissue and saline is so opaque that it is impossible to estimate the solubility of chloroform therein by direct observation as we had previously done in the case of serum, but the observations on vapour-pressure given below, show that in the case of all the tissues experimented with the solubility is much higher than in the case of water or saline.

As in the case of serum and hæmoglobin, a gradual precipitation of the tissue proteid occurs with increasing concentration of chloroform, shown by change in colour and by the emulsion of tissue becoming flocculent and separating out. This change occurs more readily, and at lower concentration of chloroform, at body temperature than at room temperature. This is shown by the fact that a mixture of the tissue and a certain strength of chloroform which remains perfectly stable and uncoagulated when kept unheated, rapidly changes colour and separates out when heated in the densimeter.

The amount of proteid in each tissue emulsion was determined by coagulation by excess of alcohol, drying and weighing; and the amount of ethereal extractive, by extracting in the manner described in the next section for preparation of ethereal extracts. The amount of ethereal extract so obtained was used to determine the amount of ethereal extract of the fresh tissue required to be added to saline in order to form an emulsion of equal concentration in lipöid for carrying out the companion vapour-pressure experiments of the succeeding section.

### *Experiment* 1.—*Brain Tissue.*

The brains of two sheep were prepared as above described. The amount of proteid in the emulsion as introduced into the densimeter was 7·36 per cent., and the amount of ethereal extractive was 4·07 per cent. The results of the experiments are given in Table I, and graphically in Curve 1, fig. 1, in which they are compared with ethereal extractive alone, of equal concentration, in emulsion in normal saline. See Experiment 5 of next section.

Table I.—Temp. 40° C.

| Percentage by weight of chloroform originally introduced. | Pressure of chloroform in vapour space, in mm. of mercury. | Percentage by weight of chloroform pumped off into vapour space. | Percentage by weight of chloroform remaining in solution. | Coefficient of distribution between vapour space and solvent. |
|---|---|---|---|---|
| 0·05 | 6·21 | 0·0038 | 0·0462 | 1 : 12·2 |
| 0·1 | 13·14 | 0·0081 | 0·0919 | 1 : 11·4 |
| 0·2 | 26·25 | 0·0162 | 0·1838 | 1 : 11·4 |
| 0·3 | 32·80 | 0·0202 | 0·2898 | 1 : 14·6 |
| 0·5 | 60·08 | 0·0370 | 0·4630 | 1 : 12·5 |
| 0·8 | 108·75 | 0·0669 | 0·7331 | 1 : 11·0 |
| 1·0 | 130·88 | 0·0806 | 0·9194 | 1 : 11·4 |
| 2·0 | 211·71 | 0·1303 | 1·8697 | 1 : 14·3 |
| 3·0 | 245·49 | 0·1511 | 2·8498 | 1 : 18·9 |
| 4·0 | 279·28 | 0·1719 | 3·8281 | 1 : 22·3 |
| 5·0 | 306·94 | 0·1889 | 4·8111 | 1 : 25·5 |

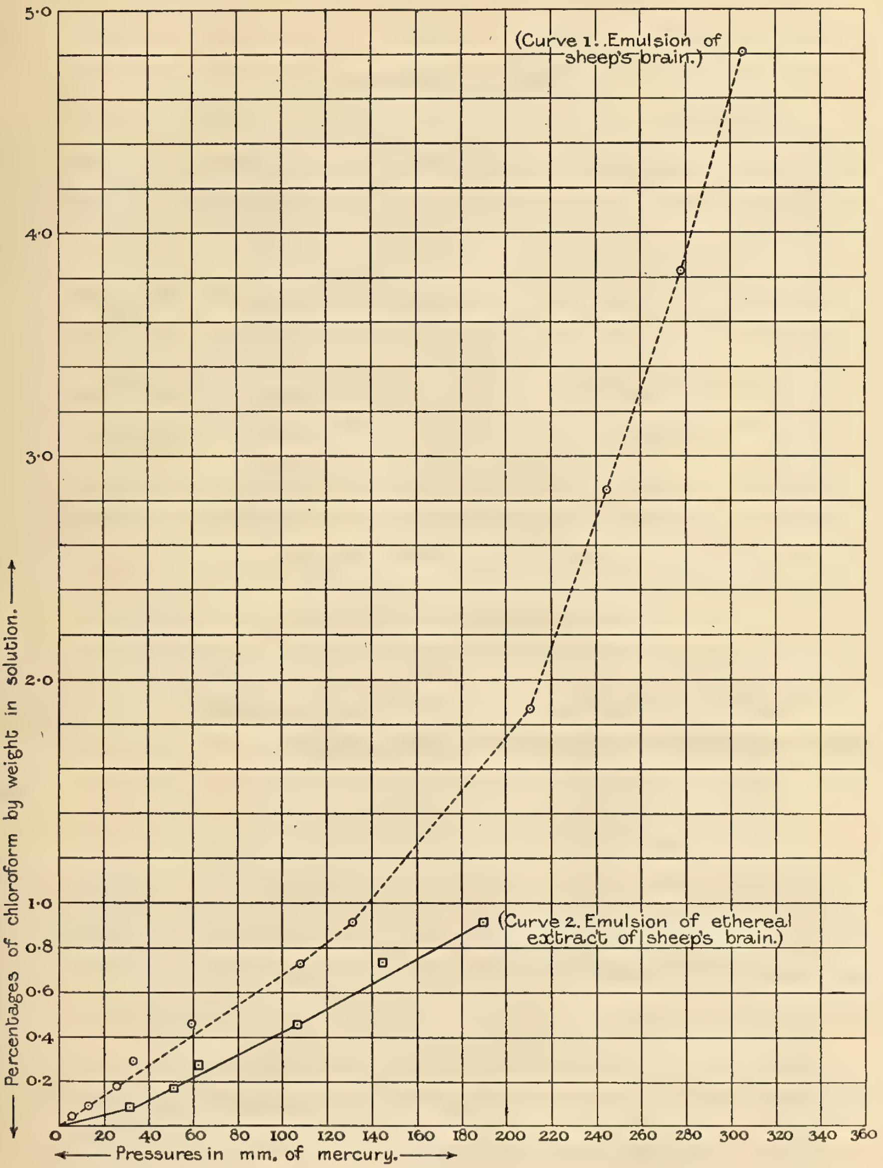

FIG. 1.

*Experiment* 2.—*Heart Muscle.*

The hearts of two sheep were perfused with saline, the parts free from visible fat were then cut out, minced and otherwise treated as above described. The percentage of proteid was 6·28 per cent., and of ethereal extractive 1·16 per cent. The results are given in Table II and graphically in Curve 1, fig. 2.

Table II.—Temp. 40° C.

| Percentage by weight of chloroform originally added. | Pressure of chloroform in vapour space, in mm. of mercury. | Percentage by weight of chloroform pumped off into vapour space. | Percentage by weight of chloroform remaining in solution. | Coefficient of distribution between vapour space and solvent. |
|---|---|---|---|---|
| 0·05 | 12·62 | 0·0078 | 0·0422 | 1 : 5·4 |
| 0·1 | 21·89 | 0·0135 | 0·0865 | 1 : 6·4 |
| 0·2 | 47·02 | 0·0290 | 0·1710 | 1 : 5·9 |
| 0·3 | 75·35 | 0·0464 | 0·2536 | 1 : 5·5 |
| 0·5 | 93·87 | 0·0578 | 0·4422 | 1 : 7·7 |
| 0·8 | 169·25 | 0·1042 | 0·6958 | 1 : 6·7 |
| 1·0 | 198·49 | 0·1538 | 0·8462 | 1 : 6·2 |
| 2·0 | 297·11 | 0·1829 | 1·8171 | 1 : 9·9 |
| 3·0 | 322·94 | 0·1988 | 2·8012 | 1 : 14·1 |
| 4·0 | 339·88 | 0·2092 | 3·7908 | 1 : 18·1 |
| 5·0 | 334·62 | 0·2060 | 4·7940 | 1 : 23·3 |

### *Experiment* 3.—*Liver Tissue.*

Sheep's liver was prepared in the usual manner. The percentage of proteid was 11·37, and of ethereal extractive 2·80. The results of the experiment are given in Table III, and graphically in Curve 2, fig. 2.

Table III.—Temp. 40° C..

| Percentage by weight of chloroform originally introduced. | Pressure of chloroform in vapour space, in mm. of mercury. | Percentage by weight of chloroform pumped off into vapour space. | Percentage by weight of chloroform remaining in solution. | Coefficient of distribution between vapour space and solvent. |
|---|---|---|---|---|
| 0·1 | 9·63 | 0·0059 | 0·0941 | 1 : 15·9 |
| 0·2 | 20·85 | 0·0128 | 0·1872 | 1 : 15·4 |
| 0·3 | 32·84 | 0·0202 | 0·2798 | 1 : 13·9 |
| 0·5 | 56·41 | 0·0347 | 0·4653 | 1 : 13·4 |
| 0·8 | 85·58 | 0·0527 | 0·7473 | 1 : 14·2 |
| 1·0 | 108·21 | 0·0666 | 0·9334 | 1 : 14·0 |
| 2·0 | 161·26 | 0·0993 | 1·9007 | 1 : 19·2 |
| 3·0 | 199·29 | 0·1227 | 2·8773 | 1 : 23·5 |
| 4·0 | 239·75 | 0·1476 | 3·8524 | 1 : 26·1 |
| 5·0 | 267·80 | 0·1649 | 4·8351 | 1 : 29·3 |

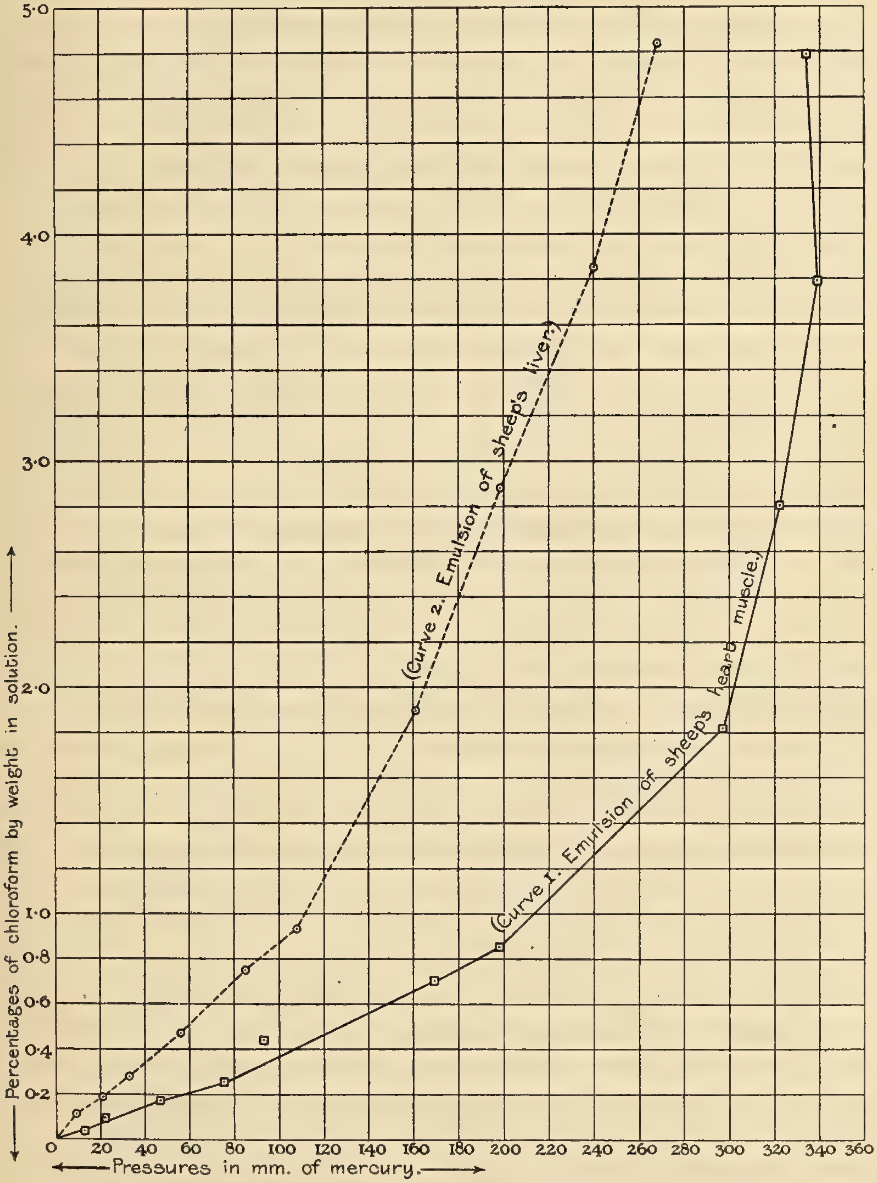
5·0
4·0
3·0
2·0
1·0
0·8
0·6
0·4
0·2
Percentages of chloroform by weight in solution.
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
Pressures in mm. of mercury.
(Curve 2. Emulsion of sheep's liver.)
(Curve I. Emulsion of sheeps heart muscle.)

FIG. 2.

### B.—*Experiments on the Relationship between Vapour-pressure and Concentration of Chloroform in Emulsions in Saline of the Ethereal Extractives (Lipoids) of Serum and Brain Tissue.*

These experiments were devised with the object of testing whether the alterations in the relationships between vapour-pressure of the anæsthetic and its concentration, as compared with water and saline, found in the case of serum and the tissues, were due entirely to lipoids or ethereal extractives contained in these fluids, or whether part of the effect was due to action of the proteids. The results shown in the curves of figs. 1 and 3, clearly show that a great deal of the action is due to the proteid.

That a certain amount of the anæsthetic will be taken up by the lipoid in a physical fashion there can be no doubt on account of the high solubility of chloroform and other anæsthetics in such lipoid substances. But we hold that the portion of anæsthetic so taken up and held by the lipoid is passive and not active, and that it is the other portion taken up by the proteid (the existence of which figs. 1 and 3 demonstrate) which is active in paralysing protoplasmic activity and producing anæsthesia.

It is a matter of common experience that the greater the amount of fatty tissue in a subject undergoing anæsthetisation, the greater the amount of anæsthetic required. The portion of anæsthetic which is absorbed by the lipoid is imprisoned as far as purposes of anæsthetisation are concerned, and so much the more anæsthetic must be given in order to raise the pressure of anæsthetic sufficiently and cause combination between cell-protoplasm and anæsthetic with resulting anæsthetisation.

The ethereal extractives (lipoids) were obtained by the following method:—The proteid of the serum or brain tissue was completely precipitated by addition of excess of absolute alcohol, and the precipitate was separated from the alcohol. The precipitate was thoroughly extracted with ether. The absolute alcohol solution was evaporated to dryness and the residue also thoroughly extracted with ether. The two ethereal extracts were united and the ether evaporated off. The total ethereal extractive was weighed and then made up into a fine emulsion by shaking with normal saline (0·75 per cent.). The volume of the emulsion was made equal to that of the serum originally taken, and in the case of the brain tissue the concentration of the emulsion of the ethereal extract was made equal to the amount of lipoid directly determined in the sheep's brain. For comparison with the results in the case of the ethereal extractive emulsion of serum, the results in the case of serum from our former paper are given, and the comparison is shown in the two curves of fig. 3. In the case of the brain tissue and the emulsion of the lipoids of brain tissue of equal concentration, the results are shown alongside in the two curves of fig. 1.

### *Separation or Coagulation of the Lipoid Emulsions by Chloroform and other Substances.*

An interesting physical effect is seen as the amount of chloroform added to the emulsion of lipoids in saline is increased. At a certain stage, dependent upon the richness of the emulsion in lipoid, a complete separation of the lipoid, in a butter-like mass, is obtained, leaving the saline practically free from lipoid. The phenomenon suggests a resemblance to the similar precipitation of proteid observed under like conditions, but there is this difference, that in the case of proteid, when the amount of anæsthetic is sufficient, the precipitate is permanently altered, being coagulated and rendered insoluble in water or saline, while the lipoid is only physically thrown out, and can be re-dissolved in ether and again made into an emulsion with saline. This phenomenon of physical aggregation of the lipoid by the anæsthetic is the more remarkable because the emulsions are exceedingly permanent and remain unaltered for days. The permanency is probably due to lecithin, and the emulsion under the microscope shows small bodies, which are not in most cases spheres, but show the appearance of bi-concave discs of varying size, many being no larger than mammalian blood-corpuscles. The physical cause for the production of such discs is at present unknown to us, but the matter is being further investigated.

In the case of the emulsion of brain tissue (containing 4·07 per cent. of lipoid) coagulation or separation of the emulsion occurs in the cold when about 2 per cent. of chloroform has been added, and the coagulation occurs much earlier at body temperature. The coagulum or separated lipoid forms a jelly-like mass, which later separates into a thin whitish fluid and a butter-like mass. These emulsions can also be coagulated or separated by solutions of neutral salts, alcohol, benzol, xylol, and other organic fluids.

The precipitation may be due to a lowering of surface tension in the emulsion, and presents an interesting analogy with the precipitation of proteids and other colloids from solution by neutral salts, chloroform, and other organic substances which act as anæsthetics. It may be noted in this connection that froth on serum disappears when chloroform is dropped into it, and we have noticed the absence of frothing on stirring on the chloroform side of our densimeter as compared with the control side.

### *Experiment 4.—Ethereal Extract of Serum.*

The extract was obtained as described, the percentage of ethereal extractive (lipoid) in the emulsion in saline as introduced into the densimeter was 0·206. The results are given in Table IV, and shown graphically in Curve 2, fig. 3, where they are contrasted with the results given by entire serum.

Table IV.—Temp. 40° C.

| Percentage by weight of chloroform originally introduced. | Pressure of chloroform in vapour space, in mm. of mercury. | Percentage by weight of chloroform pumped off into vapour space. | Percentage by weight of chloroform remaining in solution. | Coefficient of distribution between vapour space and solvent. |
|---|---|---|---|---|
| 0·1 | 38·60 | 0·0238 | 0·0762 | 1 : 3·2 |
| 0·2 | 77·41 | 0·0476 | 0·1524 | 1 : 3·2 |
| 0·3 | 108·32 | 0·0667 | 0·2333 | 1 : 3·5 |
| 0·5 | 177·71 | 0·1094 | 0·3906 | 1 : 3·6 |
| 0·8 | 262·60 | 0·1617 | 0·6383 | 1 : 3·9 |
| 1·0 | 309·27 | 0·1904 | 0·8096 | 1 : 4·3 |
| 1·2 | 351·90 | 0·2105 | 0·9895 | 1 : 4·7 |

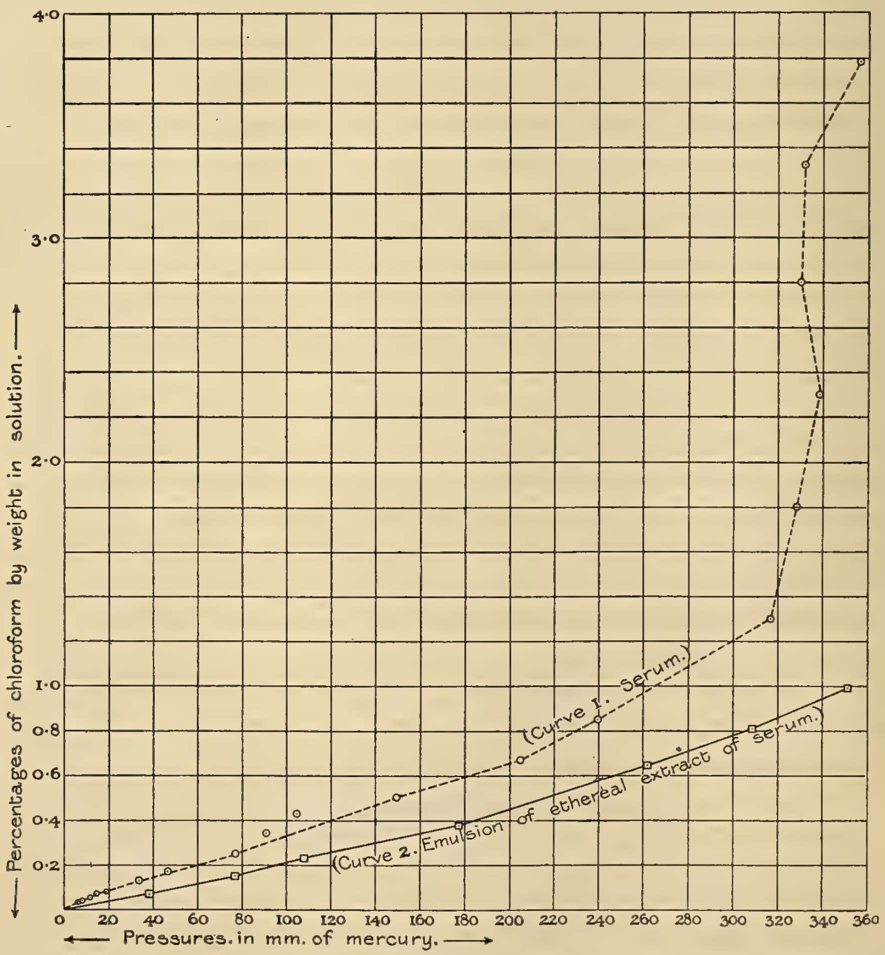

Fig. 3.

### *Experiment* 5.—*Ethereal Extract of Sheep's Brain.*

The emulsion, prepared as before, contained 4·067 per cent. of ethereal extractives. The results are given in Table V, and are contrasted graphically with the results obtained from the emulsion of the entire brain in fig. 1, Curve 1, brain tissue; Curve 2, ethereal extractives.

Table V.—Temp. 40° C.

| Percentage by weight of chloroform originally introduced. | Pressure of chloroform in vapour space, in mm. of mercury. | Percentage by weight of chloroform pumped off into vapour space. | Percentage by weight of chloroform remaining in solution. | Coefficient of distribution between vapour space and solvent. |
|---|---|---|---|---|
| 0·103 | 32·44 | 0·0199 | 0·0831 | 1 : 4·2 |
| 0·206 | 52·15 | 0·0320 | 0·1740 | 1 : 5·4 |
| 0·309 | 62·12 | 0·0381 | 0·2709 | 1 : 7·1 |
| 0·515 | 107·11 | 0·0656 | 0·4494 | 1 : 6·9 |
| 0·820 | 145·31 | 0·0890 | 0·7310 | 1 : 8·2 |
| 1·040 | 190·22 | 0·1161 | 0·9234 | 1 : 7·9 |

The experiment could not be carried beyond this point because the lipoid separated out at 40° C. at all higher concentrations in chloroform.

### C.—*Relative Solubilities of a Series of Anæsthetics in Water and Serum respectively, and the Effects of Addition of such Anæsthetics upon the Serum.*

The method of determining the solubility of the anæsthetic consisted in weighing out known amounts of the anæsthetic into water and serum respectively, stoppering the flasks, placing on the mechanical shaker for some hours, and then determining by direct observation that concentration in each case at which the anæsthetic ceased to be dissolved.

The anæsthetics so tested were chloroform, ethyl ether, ethyl acetate, amyl alcohol, amyl acetate, benzol, and xylol. The results obtained were as follows:—

#### *Chloroform.*

Solubility in water at 13° C....... 0·95 per cent.
„ serum „ ...... 4·00 „

In water, 0·8 per cent., all dissolved; 0·9 per cent., all dissolved; 1 per cent., not dissolved completely; estimated solubility, 0·95 per cent. In serum, 3 per cent., all dissolved; 3·5 per cent., all dissolved; 4 per cent., all dissolved save a few small globules; estimated solubility, almost 4 per cent.

### *Ethyl Ether.*

Solubility in water at 15° C....... 8 per cent.
„ serum „ ...... 11 „

At 10 per cent. and over in the serum there was a slight opalescence, and the fluid began to grow gelatinous; 11 per cent., completely dissolved; 12 per cent. not dissolved; and at higher percentages two phases separated, the layer on top forming a clear, thin jelly.

### *Ethyl Acetate.*

Solubility in water at 15° C....... 7·9 per cent.
„ serum „ ...... 10 „

At 8 per cent. complete solution of the ethyl acetate and commencing precipitation of the proteid occurred. Solutions of 9 and of 10 per cent. strengths also dissolved completely. Separation into two phases occurred at 12 per cent., the upper layer being viscid.

### *Amyl Alcohol.*

Solubility in water at 15° C....... 2·4 per cent.
„ serum „ ...... 8 „ or over.

At 3 per cent. complete solution of anæsthetic with commencing precipitation of proteid. This precipitation gradually increased with concentrations of 3·5 and 4 per cent. up to 8 per cent., when a thick cream was formed. A separation into two phases could not be observed at any stage.

### *Amyl Acetate.*

Solubility in water at 15° C....... 0·25 per cent.
„ serum „ ...... 1·5 „

At 1 per cent., complete solution of anæsthetic with precipitation of proteid; at 1·5 per cent., all dissolved except a few minute globules.

### *Benzol.*

Solubility in water at 15° C....... 0·15 per cent.
„ serum „ ...... 0·6 „

At 0·5 per cent., commencing precipitation and opalescence; 0·6 per cent. completely dissolved; 0·7 per cent., not all dissolved.

### *Xylol.*

Solubility in water at 15° C....... 0·016 per cent.
„ serum „ ...... 0·2 „

At 0·1 per cent., complete solution, cloudy from proteid precipitation; 0·2 per cent., all dissolved; 0·3 per cent., not all dissolved.

In all cases the solubility in serum is higher than in water, and it is clear from the results that a similar association between the anæsthetic and the proteid of the serum occurs throughout the series. The amount of ethereal extractive in the different samples of serum used for the determinations varied between 0·24 and 0·36 per cent., thus showing that the increased solubilities found could not be due to solution of the anæsthetic in lipoid or ethereal extractives present in the serum.

### D. *Changes in Depression of the Freezing Point of Water, Saline, and Serum respectively caused by the addition of Chloroform.*

These experiments were undertaken to elucidate if possible the state in which the chloroform existed in the proteid solutions.

The determinations were made with Beckmann's apparatus. On account of the chloroform being volatile, the apparatus had to be completely closed, and the stirring accomplished by electro-magnet in the usual way. The solution was placed in a straight tube, which was almost completely filled, so that no correction was required for escape of chloroform into the air space above the fluid.

The changes in depression of freezing point are small, but in every case the results are comparable and concordant. In the table (Table VI) the percentages of chloroform are given by weight, and the figures are corrected to allow for the degree of supercooling.

Table VI.—Changes in $\Delta$ for Amounts of Chloroform added to Various Solutions indicated at the Heads of the separate Columns.

| Distilled water plus 0·6 per cent. chloroform. | Saline solution (0·75 per cent.) plus 0·6 per cent. chloroform. | Serum plus 0·6 per cent. chloroform. | Serum plus 1·0 per cent. chloroform. | Serum plus 2·0 per cent. chloroform. | Serum plus 3·0 per cent. chloroform. | Serum plus 4·0 per cent. chloroform. |
|---|---|---|---|---|---|---|
| 0·061 | 0·081 | 0·060 | 0·101 | 0·135 | 0·138 | 0·142 |
| 0·075 | 0·075 | 0·062 | 0·086 | 0·141 | — | — |
| 0·077 | 0·072 | 0·072 | 0·104 | — | — | — |
| 0·099 | 0·082 | — | — | — | — | — |
| Average 0·078 | 0·078 | 0·065 | 0·097 | 0·138 | 0·138 | 0·142 |

It is noticeable from the table (1) that the lowering of freezing point for chloroform in serum is less than the lowering due to the same amount of chloroform in water or saline; (2) that the lowering of freezing point in the case of serum goes on increasing far beyond the point of maximum solubility of chloroform in water or saline, but that the increased lowering is not strictly proportional to the extra amount of chloroform added, but progressively less; and (3) above 2 per cent. further addition of chloroform has no more effect upon the freezing point. The latter fact points to a removal of the chloroform by the proteid corresponding to the steep rise in the vapour-pressure curves at high concentrations.

### E. *Changes in Electrical Conductivity in Saline and Serum produced by the addition of Chloroform.*

The electrical conductivities were determined by the method of Kohlrausch, at the temperatures of 0°, 15°, and 40° C. In the case of saline (0·75 per cent.) and different samples of serum, both before and after addition of definite weighed amounts of chloroform. In the case of the saline the amount of chloroform added was 0·6 per cent. by weight, and in the case of serum amounts of 0·6, 1, 2, and 3 per cent. by weight were added in successive experiments. On account of variations in the different samples of serum there are considerable fluctuations in the results, but certain facts are observable. In Table VII the alterations in the value of K (the specific conductivity, multiplied by $10^2$) are shown, which result from the addition of the amounts by weight of chloroform shown at the head of each column. It is noticeable that the conductivity is reduced without exception in all cases where chloroform is added to normal saline, due to a diminution of the ionisation of the sodium chloride by the chloroform and alteration of ionic velocity. But when chloroform is added to serum there is, in many cases, an actual increase in conductivity, and in other cases the diminution is less than occurs with the corresponding amount of chloroform added to normal saline. This indicates that there is a tendency for inorganic salts to be set free from the proteid and add to the conductivity. This is only seen as an actual increase where it outbalances the action above mentioned of the chloroform in reducing conductivity in pure saline solution.

The table shows that the amount of increase in conductivity varies with the temperature and the amount of chloroform added. Thus at 0° C. the diminution in conductivity is the same in saline and serum for 0·6 per cent. of chloroform, but when 1 per cent. of chloroform has been added, the diminution becomes converted into an increase; again, at higher percentages, a diminution is seen, as if all the electrolyte possible had been detached from the proteid at the lower concentrations, and the diminution was now that due to the effect of the additional chloroform upon the saline in solution (fig. 4).

Table VII.—Changes in $K \times 10^2$ for amounts of Chloroform added to Serum and Saline.

| Temperature. | Saline solution (0·75 per cent.) plus 0·6 per cent. chloroform. | Serum plus 0·6 per cent. chloroform. | Serum plus 1·0 per cent. chloroform. | Serum plus 2·0 per cent. chloroform. | Serum plus 3·0 per cent. chloroform. |
|---|---|---|---|---|---|
| ° C. | | | | | |
| 0 | − 0·0140 | − 0·0267 | + 0·0015 | − 0·0275 | − 0·0176 |
| 0 | − 0·0204 | − 0·0077 | + 0·0160 | − 0·0139 | − 0·0275 |
| Average ... | − 0·0172 | − 0·0172 | + 0·0087 | − 0·0207 | − 0·0225 |
| 15 | − 0·0360 | + 0·0390 | + 0·0440 | + 0·0140 | − 0·0060 |
| 15 | − 0·0020 | + 0·0460 | + 0·0890 | − 0·0050 | − 0·0130 |
| 15 | − 0·0361 | + 0·0032 | − 0·0146 | − 0·0255 | − 0·0336 |
| 15 | − 0·0200 | − 0·0092 | − 0·0044 | − 0·0162 | − 0·0323 |
| Average ... | − 0·0235 | + 0·0197 | + 0·0285 | − 0·0082 | − 0·0212 |
| 40 | − 0·0270 | − 0·0240 | − 0·0320 | − 0·0410 | — |
| 40 | − 0·0300 | − 0·0270 | − 0·0880 | − 0·0690 | — |
| 40 | − 0·0410 | − 0·0140 | − 0·0470 | − 0·0650 | — |
| 40 | − 0·0399 | − 0·0170 | − 0·0331 | − 0·0577 | − 0·0674 |
| 40 | − 0·0314 | − 0·0061 | − 0·0312 | − 0·0669 | − 0·0488 |
| Average ... | − 0·0339 | − 0·0176 | − 0·0463 | − 0·0599 | − 0·0581 |

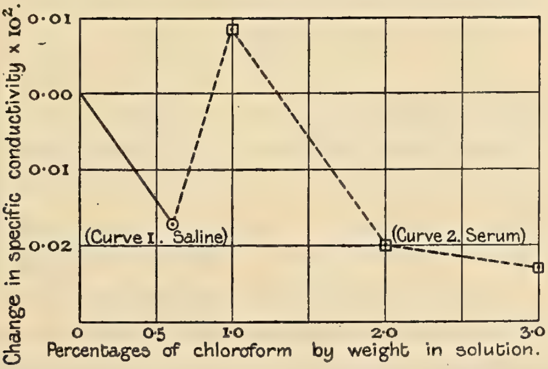

Fig. 4.—Changes in Electrical Conductivity on addition of Chloroform to Saline (Curve 1) and Serum (Curve 2) at 0° C. Between the first two points the curves are identical.

The effects are most marked at 15° C.; here, instead of the diminution occurring on addition of chloroform to pure saline, there is found an increase in conductivity in the case of serum both for addition of 0·6 per cent. and 1 per cent., which as before becomes converted into a diminution for 2 per cent. and 3 per cent. (fig. 5). At 40° C. there is no actual increase at any concentration, but the fall in conductivity is less for serum than saline at 0·6 per cent., and this fall is increased at the higher concentrations in chloroform (fig. 6).

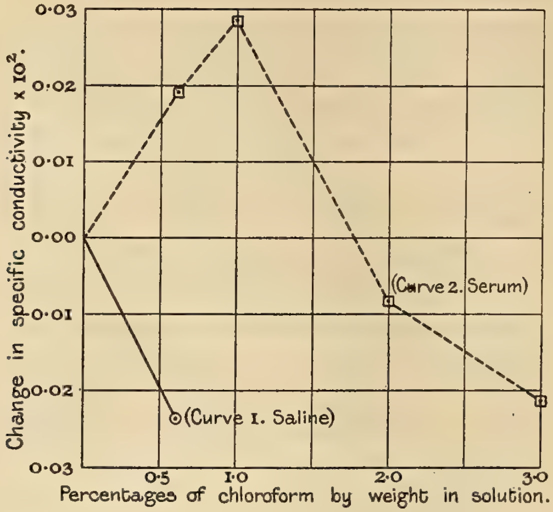

Fig. 5.—Changes in Electrical Conductivity on addition of Chloroform to Saline (Curve 1) and Serum (Curve 2) at 18° C.

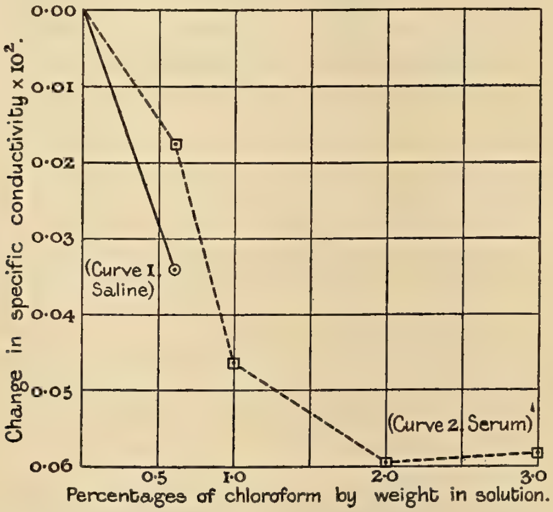

Fig. 6.—Changes in Electrical Conductivity on addition of Chloroform to Saline (Curve 1) and Serum (Curve 2) at 40° C.

Taking the experiments as a whole, there is evidence of two opposing factors partially masking each other, the first being due to a setting free of electrolyte from the proteid by the added chloroform increasing the conductivity, and the second the well-known action of a non-electrolyte in diminishing the conductivity, which is seen clearly in the case of the pure saline plus chloroform.

### *Summary and Conclusions.*

The experiments recorded in the present communication support the conclusion drawn in our previous paper that anæsthetics form unstable compounds or aggregates with the proteids of the tissue cells, and that anæsthesia is due to a paralysis of the chemical activities of the protoplasm as a result of the formation of such aggregations.

The comparative experiments with ethereal extracts demonstrate that the action is upon the cell proteids and not upon the lipoids.

The compounds or aggregations so formed are unstable, and remained formed only so long as the pressure of the anæsthetic in the blood is maintained.

The results of our experiments may be summarised as follows:—

(1) The solubility of all anæsthetics experimented with is higher in serum than in water.

(2) At a certain concentration, definite for each anæsthetic, there occurs opalescence and commencing precipitation of proteid.

(3) At equal concentration of chloroform in water or saline on the one hand, and serum, hæmoglobin, or the tissues (brain, heart, muscle, and liver) on the other, the vapour-pressure is always higher in the former than in the latter.

(4) The curve connecting vapour-pressure and concentration is, in the case of water and saline, a straight line; while in the case of serum, hæmoglobin, and the tissue proteids it is a curve showing association, especially at the higher concentrations.

(5) Comparative determinations of vapour-pressure and concentration, in serum and brain tissue and in ethereal extracts of these equal in concentration of lipoid, show that the proteid of the tissue combines with the anæsthetic.

(6) Determinations of the effects of addition of chloroform upon the lowering of freezing point confirm the results obtained by the vapour-pressure and solubility determinations.

(7) Determinations of the changes in electrical conductivity caused by addition of chloroform indicate that accompanying the combination of the anæsthetic with the proteid there takes place a splitting off of electrolytes.

(8) When the lipoids, extracted from serum or tissues by ether are made up into an emulsion with normal saline, many of the lipoids take the form of bi-concave discs.

(9) The lipoid emulsions are very permanent, but separate on the addition of anæsthetics or neutral salts, in similar fashion to colloidal solutions.

# *On the Effects of Alkalies and Acids, and of Alkaline and Acid Salts, upon Growth and Cell Division in the Fertilized Eggs of* Echinus esculentus.—*A Study in Relationship to the Causation of Malignant Disease.*

By Benjamin Moore, M.A., D.Sc., Johnston Professor of Bio-Chemistry, University of Liverpool; Herbert E. Roaf, M.D. Toronto, Johnston Colonial Fellow, University of Liverpool; and Edward Whitley, M.A. Oxon.

(Communicated by Professor W. A. Herdman, F.R.S. Received October 9,—Read November 23, 1905.)

The results of observations previously made in the Bio-chemical Laboratory of the University of Liverpool have shown that free hydrochloric acid is absent from the gastric contents, or greatly reduced, in nearly all cases of malignant disease, *no matter where the malignant growth happens to be situated.*

In the paper describing these observations it was pointed out that the most probable cause of this absence of the free hydrochloric acid was an increased alkalinity of the blood-plasma, as a result of which the hydrogen ion concentration in the plasma was so far reduced that the oxyntic cells were no longer able to separate an acid secretion from it.*

It seemed to us, therefore, desirable to test the effects of alterations in the concentration of hydrogen and hydroxyl ions respectively, upon the growth and cell division in some organism in which cell division was proceeding rapidly, and which could be easily subjected directly to changes in acidity or alkalinity of the medium in which it was living.

We selected for this purpose the fertilized eggs of a species of sea-urchin (*Echinus esculentus*), because at the season of the year at which our experiments were carried out (April, 1905) the ripe eggs can readily be obtained from the gonads of the female, and be fertilized by mixing with the sperm similarly obtained from a ripe male. Hence from the single cell stage onward the rate of growth and any irregularities in cell division can be observed, and the effects of the addition of alkali or acid, or alkaline or acid salt, can be compared under exactly similar conditions, and contrasted with a control grown alongside in unaltered sea-water.

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* 'Roy. Soc. Proc.,' B, vol. 76, p. 138, 1905.

The experiments were made, by kind permission of Professor W. A Herdman, F.R.S., at the Marine Biological Station, Port Erin, Isle of Man, during the month of April, 1905.

J. Loeb,* in 1898, reported experiments on the influence of acids and alkalies on the development of the larvæ of sea-urchins, showing that the addition of acids to the sea-water delayed the development, and finally, at a certain concentration of hydrogen ions, inhibited the development completely. With the addition of alkalies the development during the first 12 hours was not at all hastened, or hastened to a hardly appreciable extent. On the second day, however, and sometimes also even on the third day, the eggs that developed in sea-water which had been made alkaline were occasionally, but not always, in advance of eggs of the same brood which had been raised in normal sea-water.

At the time of the publication of this paper, Loeb held the current view that sea-water had an alkaline reaction, and accordingly drew the conclusion that, for developmental processes, it is necessary to have an alkaline reaction or, in other words, a higher concentration of hydroxyl ions than exists in distilled water.

At a later date Loeb,† however, came to the conclusion that normal sea-water must be regarded as a practically neutral solution, and hence ascribed the favouring action of alkalies upon cell-division and growth to the neutralizing action of the alkali added upon acid products formed by the cells in the process of growth which would otherwise cause an accumulation of hydrogen ions and arrest the development.

This opinion is based by Loeb upon the results of the observations of Friedenthal,‡ Fraenkel,§ and Farkas,‖ which indicate that blood-plasma possesses no higher concentration in hydroxyl ions than distilled water; upon observations made by Dr. Cottrell at Loeb's suggestion upon the sea-water of the Bay of San Francisco by means of hydrogen electrodes, which showed

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[*] 'Arch. f. Entwickelungsmechanik d. Organismen,' 1898, vol. 7, p. 631.

[†] 'Arch. f. d. ges. Physiol.,' 1903, vol. 99, p. 637; *ibid.*, 1904, vol. 101, p. 340; *ibid.*, 1904, vol. 103, p. 506; 'Univ. of California Publications, Physiology,' 1903—4, vol. 1, pp. 39, 139.

[‡] 'Arch. f. Anat. u. Physiol., Physiol. Abth.,' 1903, p. 550.

[§] 'Arch. f. d. ges. Physiol.,' vol. 96, 1903, p. 601.

[‖] 'Arch. f. d. ges. Physiol.,' vol. 98, 1903, p. 551; see also Höber, 'Arch. f. d. ges. Physiol.,' vol. 99, 1903, p. 572.

that the concentration of hydrogen ions was greater by one power of ten than in distilled water; and upon the observations of Loeb himself, (1) that normal sea-water is practically neutral to phenol-phthalëin, and (2) that addition of sodium bicarbonate or of di-sodic phosphate, which from their neutral reaction to phenol-phthalëin are regarded by Loeb as neutral salts, produced the same favouring effect upon cell-division and reproduction as the caustic alkalies.

A solution of the mixed phosphates or carbonates in which there is an approximate balance between the concentration of hydrogen and hydroxyl ions such that these concentrations are nearly equal, cannot, however, be regarded as neutral in the same sense as distilled water is neutral; or as being acid or alkaline in the same sense as a solution containing only free acid or free alkali can be regarded as being acid or alkaline.

Nor will such a solution of phosphates and carbonates as is present in blood-plasma or sea-water have a similar action upon living cells to either distilled water or a neutral solution of such salts as sodium chloride of equal osmotic concentration.

Such solutions form a peculiar type of their own, which possess the property of behaving either as an acid or as a base. Such solutions can behave in this manner to any substances possessing weak acid or basic properties brought into the same solution, and it is for this reason that such solutions behave entirely differently to distilled water in their associating or dissociating action upon coloured indicators, and so indicate at the same time an acid reaction to one indicator and an alkaline to another.

A quantity of acid or of alkali can be added to the solution, which would cause an enormous variation in the relative concentration of hydrogen and hydroxyl ions if added to distilled water, without altering to anything like the same extent the relative concentrations of these two ions.

Therefore blood-plasma, and to a less extent sea-water, possess, on account of the mixed phosphates and carbonates which they contain, a steadying action upon variations in the concentrations of the hydrogen and hydroxyl ions. When acid or alkali is added to the plasma, instead of there occurring a corresponding swing in the concentration of the hydrogen and hydroxyl ions, there takes place an alteration in the equilibrium of the ions of the phosphates and carbonates, which neutralizes, in great part, the hydrogen or hydroxyl ions added, and prevents the plasma becoming markedly acid or alkaline. Without such a controlling action the life of the cells would be rendered impossible, for, as our experiments show, the living cell is most sensitive to even small variations in either hydrogen or hydroxyl ion.

This powerful action of alterations in concentration of hydrogen or hydroxyl ion, arises from the fact that the constituents of the protoplasm behave like very weak acids or bases, and are affected by variations in hydrogen or hydroxyl ions in a similar manner to coloured indicators. Very small variations in ionic concentrations compared to those found in even dilute solutions of free acid or alkali, will hence cause the constituents of the cell to become almost completely associated or dissociated, and prevent the chemical reactions from occurring which are necessary for the metabolism and life of the cell.

This is shown in our experiments by the very small amounts of either acid or alkali which suffice to kill the cell. Within the limits at which life is still possible, but at which the concentrations of the two ions are varied from the normal, profound alterations occur both in the rate of growth, and in the type of the cell divisions.

In the case of malignant growths similar variations in hydrogen and hydroxyl ions occur as shown by the absence of the acid in the gastric juice.

It is from this point of view that we have studied the effects upon growth and cell-division of the addition of acids or alkalies in small amounts to the medium in which such processes are taking place.

The problem before us is that of the effects upon the cell of variations in the hydrogen and hydroxyl ion concentration, and for the purposes of such an enquiry it is not neccessary to know what are the *actual* concentrations of the two ions in normal plasma, nor to discuss which of these two magnitudes is the greater.

If alkali be added to plasma or to sea-water the concentration in hydrogen ions will fall and that in hydroxyl ions will rise, and conversely on adding acid a reverse change will take place. The experiments we have made show the results of such changes upon cell growth and nuclear division.

A number of other observers have drawn attention to irregularities in cell growth and nuclear division induced by the action of foreign chemical substances upon the dividing cells.

Thus, O. and R. Hertwig,* and also Galleotti† have shown that pathological mitoses with irregular division in number and amount of the chromosomes may be induced by such substances as quinine, chloral, nicotine, anti-pyrine, cocaine, and potassium iodide.

The derangements in division so produced closely resemble the pathological divisions described in cancer cells by Klebs,‡ Hansemann,§ and Galleotti.‖

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* 'Jenaische Zeitsch. f. Naturwissenschaft,' vol. 20, 1887, pp. 120, 477.

† 'Beit. z. path. Anat. u. z. allgem. Path.,' vol. 14, 1893, p. 288.

‡ 'Die allgem. Pathologie,' Fischer, Jena, 1899, Part 2, pp. 523, *et seq.*

§ 'Virchow's Archiv,' vol. 119, 1890, p. 299 ; vol. 123, 1891, p. 356.

‖ *Loc. cit.*

Hansemann recognizes in such abnormal mitoses two general groups, (1) *asymmetrical mitoses*, in which the chromosomes are unequally ditributed to the daughter cells, and (2) *multipolar mitoses*, in which the number of centrosomes is more than two, and more than one spindle is formed.

Hansemann and Galleotti find in this asymmetry of mitoses an explanation of the well-known fact that in cancer cells many of the nuclei are especially rich in chromatin (hyperchromatic cells) while others are abnormally poor (hypochromatic cells). According to Galleotti, the asymmetrical mitoses which may be artificially produced in the epithelial cells of salamanders by treatment with dilute solutions of anti-pyrine, cocaine or quinine are exactly like those seen in carcinoma.

In these experiments, the drugs used for inducing pathological mitoses are not such as can occur in carcinoma. A variation of the concentration in hydrogen and hydroxyl ions can, however, occur in the plasma, and, in fact, the observations on the absence of acid in the gastric secretion in cases of carcinoma make it probable that such variations do occur.

It is hence of interest that in our experiments detailed below, in which the concentration of hydrogen and hydroxyl ions was artificially varied, we have found that with increased alkalinity, at a point just short of that at which cell growth was stopped, such pathological mitoses do occur.

We have observed both the asymmetrical mitoses with unequal distribution of chromosomes and only two centrosomes, and the multipolar mitoses with three or more centrosomes. Also, as the alkali was increased above the normal of sea-water, a marked tendency to irregularity in size and shape of the resulting cells was observed similar to that seen in cancer cells.

As the amount of alkali was increased, there occurred also a shortening of the dividing chromatin rods, similar to that seen in most maturation divisions, until the rods became in some cases converted into rounded dots. In a certain percentage of the divisions, the number of chromosomes was reduced. The number of chromosomes is exceedingly difficult to count with certainty, but the reduction in many cases amounted approximately to one-half the normal.

### *Experimental Methods.*

We have investigated in our experiments the effects of the following alkalies and acids, and alkaline and acid salts :—

Sodium hydrate, potassium hydrate, calcium hydrate, ammonia, hydrochloric acid, acetic acid, carbonic acid, sodium carbonate, sodium bicarbonate mono-sodium phosphate ($NaH_2PO_4$), and di-sodium phosphate ($Na_2HPO_4$).

The stock solutions of the acids and caustic alkalies were made in distilled water, titrated and standardized to normal strength, and from these decinormal solutions in distilled water were prepared. The carbonates and monosodic phosphate were made up in decimolecular strength, and the di-sodic phosphate in $\frac{1}{20}$ molecular strength, on account of its lower solubility. A saturated solution of calcium hydrate was prepared in distilled water decanted from the lime, and standardized against decinormal acid, and this standardized solution was used in small measured amounts for addition to the sea-water containing the samples of eggs as in the other cases. Similarly, in the case of the carbonic acid, a comparatively strong solution of carbonic acid was prepared by passing the gas through sea-water; this solution was standardized at once with decinormal alkali, using phenol-phthalëin as indicator, and immediately added in appropriate small measured quantities to the various measured sea-water and egg-mixtures.

It may be pointed out that there was no appreciable variations in osmotic pressure of the sea-water caused by the addition of the reagent solutions, because the volume added was small in comparison with the volume of sea-water and egg-mixture to which it was added; further the decinormal solutions added, though hypotonic, do not lie very far below the molecular concentration of the sea-water. If the decinormal solutions had been made in sea-water instead of distilled water, an equal or greater amount of change in osmotic pressure would have resulted on account of their being hypertonic, and also precipitation of constituents of the sea-water would in certain cases have occurred. The change in osmotic pressure was, however, in all cases quite a negligible quantity.

The experiments involved the use of a large number of vessels on account of the long series of mixtures of sea-water and fertilized eggs with their varying amounts of added chemicals of different kinds, and we had not anticipated this heavy demand when starting upon our expedition. Hence, we had to make use of such materials as we could find in the Marine Biological Station, or obtain at Port Erin. The earlier experiments were made in well washed out glass jam-pots such as are used for collecting fresh marine specimens, but later we found ordinary plain tumblers of the usual size most convenient.

The amount of surface for aeration compared to the volume of fluid was the same in each case throughout each series of experiments, and hence the results obtained are strictly comparable with one another.

The method of procedure in starting an experiment was as follows:—The shells of a number of Echini were cut open circularly so as to expose the uninjured gonads, until a ripe male and a ripe female had been obtained as shown by examining under the microscope, and ascertaining that the spermatozoa were active, and the eggs of mature size and well formed.

Usually two gonads from the female, and about half to one gonad from the male were taken, each in a separate tumbler, gently rubbed up with a small quantity of sea-water, and then separated from *débris* by filtering through a coarse piece of muslin. The eggs were somewhat diluted with sea-water, a quantity of sperm added, and a drop of the mixture taken out and examined with the microscope until it was ascertained that the eggs had developed their fertilization membranes.

A number of tumblers corresponding to the number of controls and the total of the various dilutions of the different chemicals to be tested for their effects upon the growth of the eggs had previously been arranged and numbered. The mixture of fertilized eggs was now diluted to a larger volume with sea-water so as to afford 200 c.c. for each tumbler, and this volume was measured out into each of the tumblers. As rapidly as possible the desired amount of each chemical was added in each case, and the time of starting noted. The progress of development was observed and noted a few hours after starting the experiment, again the following morning, and so on.

In a few cases instead of diluting the egg-mixture after fertilization to a large volume, the proper amount of sea-water was measured out into each tumbler, then an equal volume of the egg-mixture was added to each, followed by the desired amount of the chemical solution.

For the purpose of examining the progress of development a dip was taken out by means of a small pipette into a watch-glass and examined under a low power of the microscope. The state of development of the growing embryo was noted especially with regard to relative rate of growth in presence of the various strengths of the different alkaline and acid solutions. The number of cells was counted in the earlier stages, or the number of cells in an optical section of the circumference of the blastulæ in the later stages; the commencement of ciliary motion was noted, and the stages in the development of the gastula in those cases where the larvæ developed so far. Also any irregularities in shape and size of cells in the different cases were noted.

In certain cases, the progress of any change in chemical reaction was noted by adding indicators in parallel experiments carried out alongside. Interesting results as to the action of the indicators themselves were so obtained. It was found that the reaction to "di-methyl" did not change throughout the experiment, but the reaction to phenol-phthalëin, which was faintly alkaline even in normal sea-water at the commencement of the experiment, slowly changed towards the acid side. As it was found, however, that phenol-phthalëin even in very small amounts inhibited and caused irregularities in cell-division, the method was adopted of testing quantitatively by titration the reaction to both phenol-phthalëin and "di-methyl" at the end of each experiment after growth had stopped. It was found that even where alkali had been added at the commencement of the experiment the reaction to phenol-phthalëin had become slightly acid at the end in most experiments. Since the amount of alkalinity to "di-methyl" had not altered throughout the experiment, but gave in all cases very approximately the alkalinity of sea-water plus or minus the alkali or acid artificially added, it follows that the change in reaction to phenol-phthalëin must be due to a very weak acid given off during the development of the embryos. Since carbon-dioxide produces exactly the same effects, and respiration must occur in the process of development, it is almost certain that the change in reaction must be due to production of carbonic acid. On account of this natural production of carbonic acid, the results of the first twenty-four hours' growth are the most valuable, because here the alkaline reaction to phenol-phthalëin persisted, and the production of carbonic acid was small.

After preliminary experiments had determined the range at which the developing embryos were definitely affected by the different chemicals, a few final experiments were made in which only two or three concentrations of each chemical were included lying at about the proper range, and the last such experiment was interrupted when the more advanced sets of embryos had reached the morula or early blastula stage.* The embryos were fixed in Flemming's and Hermann's fluids, embedded and cut in paraffin, and stained for nuclear division by Heidenhain's iron-alum and hæmatoxylin staining, following Flemming's description. The drawings illustrating the paper were made from this series of preparations, with the exception of fig. 18, which was drawn from the fresh growing cells.

*Reaction of Sea-Water of Port Erin Bay.*—One hundred cubic centimetres of sea-water was taken and titrated against decinormal caustic soda solution, (*a*) with di- methyl- amido- azo- benzol as indicator, and (*b*) with phenol-phthalëin as indicator. With the "di-methyl" in a first trial 2·35 c.c. were required, in a second 2·37, therefore the alkalinity was = 0·00236 normal. With the phenol-phthalëin 0·24 c.c. and 0·22 c.c. were required, alkalinity = 0·00023 normal.

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* After cutting sections, it was found that many of the cells described in Table XI (pp. 123, 124) in the fresh condition as morulæ really possessed in section a small central cavity, and so are termed blastulæ in the descriptions of the drawings. The organisms described as blastulæ in the tables showed in the fresh condition an outer layer of cells.

### EXPERIMENTS WITH

### Table I.—

### Experiment No. 1,

| No. | Amount of sodium hydrate in decinormal solution, added to 200 c.c. of sea-water. | Amount of added alkali in the solution, expressed as a fraction of normal strength. | Observations of condition | |
|---|---|---|---|---|
| | | | 4 hrs. | 17 h. 50 m. |
| 1 | Control | — | Four-celled stage | Blastulæ ............ |
| 2 | 0·2 c.c. | 0·0001 | Four-celled stage | Blastulæ ............ |
| 3 | 0·4 „ | 0·0002 | Four-celled stage | Blastulæ ............ |
| 4 | 0·8 „ | 0·0004 | Four-, six-, and eight-celled stages. Irregular divisions | Blastulæ. Further advanced in cell-division than control |
| 5 | 1·4 „ | 0·0007 | Four-celled stage. Irregular division of cells | Blastulæ. Further developed than control |
| 6 | 2·0 „ | 0·0010 | Majority two-celled. A few four-celled. | Blastulæ. Further developed than control |

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* In order to distinguish throughout between different sets of eggs, each lot of fertilized

It is to be noted in this experiment that there is a distinct favouring action of the added
stages of No. 6, but not in sufficient amount to stop growth (see Experiment 2), and as the alkali
Also Nos. 4, 5 and 6 remain alive and develop further than Nos. 1, 2 and 3.

No. 5 was, at the end of the period 91 h. 50 m., divided into two portions of 100 c.c. each,
it. The subsequent development of 5*b*, which had the alkali added, was much more rapid than

CAUSTIC ALKALIES.

Sodium Hydrate.

Brood* of Eggs No. 1.

| of embryos, at interval after start of experiment given at head of each column. | | | | | |
|---|---|---|---|---|---|
| 24 h. 50 m. | 29 h. 15 m. | 41 h. 45 m. | 70 h. 30 m. | 91 h. 50 m. | 114 h. |
| Blastulæ. A few moving | Blastulæ. All in active motion | Blastulæ. All in active motion | Commencing gastrulæ. Nearly all dead | All dead, and degenerating | — |
| Blastulæ. A few moving | Blastulæ. All in active motion | Blastulæ. All in active motion | Commencing gastrulæ. Nearly all dead | All dead, and degenerating | — |
| Blastulæ. A few moving | Blastulæ. All in active motion | Blastulæ. All in active motion | Commencing gastrulæ. All dead | — | — |
| Blastulæ. A few moving | Blastulæ. All in active motion | Blastulæ. All in active motion | Well-developed gastrulæ. All active | Late gastrulæ. Very active (glass broken by accident) | — |
| Blastulæ. Motionless | Blastulæ. All in active motion | Blastulæ. All in active motion | Well-developed gastrulæ. All active | Late gastrulæ. Not so far developed as No. 4 | Forming plutei |
| Blastulæ. Motionless | Blastulæ. All in active motion | Blastulæ and some gastrulæ. All in active motion | Well-developed gastrulæ. All active | Late gastrulæ. (Formalin added by mistake) | — |

eggs is numbered as belonging to the same brood.
alkali in the earlier stages, especially observable in No. 4; the alkali is in excess in the earlier becomes diminished by the $CO_2$ given off in growth, No. 6 increases and passes the control.

of which the first (5*a*) was left unaltered, while the second had 0·5 c.c. of N/10 NaOH added to that of 5*a*, and the embryos remained alive much longer.

## Table II.

Experiment No. 2, Brood No. 2. The previous experiment having shown that the maximum amount of sodium hydrate necessary to stop development had not been added in Experiment 1, in this experiment larger amounts of alkali were added, but it is to be noted that the amount of alkali is still excessively low.

| Number. | Amount of sodium hydrate in decinormal solution, added to 200 c.c. of sea-water. | Amount of added alkali in the solution, expressed as a fraction of normal strength. | Observations of embryos at stated times after commencement of experiment. | | |
|---|---|---|---|---|---|
| | | | 5 hrs. | 17 h. 30 m. | 24 h. 30 m. |
| 1 | Control | — | Two-celled stage …………………… | Morulæ and early blastulæ (17 cells in circumference of optical section) | Blastulæ; dead |
| 2 | 3 c.c. | 0·0015 | Nearly all single-celled, but several two-celled, and occasional irregular four-celled | One or two morulæ; others containing a dozen or less unequally-sized cells in capsule | A few morulæ; no blastulæ |
| 3 | 4 ,, | 0·0020 | Nearly all single-celled; but somewhat more two-celled than in No. 2, with the two cells very unequal in size | Single cells, in a gelatinous-looking debris. Cell substance showing regular reticulation. One two-celled and one three-celled group seen | No further development |
| 4 | 5 ,, | 0·0025 | All single cells in a gelatinous mass | All single reticulated cells in gelatinous debris, except one partially divided into two, and one six-celled group | No further development |

Development came to an end in the control of this series earlier than in Experiment 1, but the contrast between the control and the others is obvious.

## Table III.

### Experiment No. 3, Brood No. 7.

| Number. | Amount of sodium hydrate in decinormal solution, added to 200 c.c. of sea-water. | Amount of added alkali in the solution, expressed as a fraction of normal strength. | Observations of embryos at stated times after commencement of experiment. | | | |
|---|---|---|---|---|---|---|
| | | | 19 h. 45 m. | 47 h. 45 m. | 66 h. 30 m. | 94 hrs. |
| 1 | Control | — | Well-developed blastulæ | Gastrulæ in active movement | Gastrulæ. Less advanced than No. 2 | Well-developed gastrulæ. Archenteron completed |
| 2 | 1 c.c. | 0·0005 | Well-developed blastulæ. More advanced than control | Gastrulæ in very active movement | Gastrulæ. More advanced than control | More advanced gastrulæ, with arms commencing to form |
| 3 | 2 „ | 0·0010 | No blastulæ. Morulæ at different stages, many very early | Blastulæ not yet moving | Majority in early gastrula stage | Early gastrulæ, with archenteron only commencing |
| 4 | 3 „ | 0·0015 | Half in two-celled stage only. Remainder countable, and very early morulæ. Less advanced by far of the series | Two-celled, and early morulæ | Dead and degenerating. None have passed early morula stage | — |

Table IV.—Potassium Hydrate.

Experiment 1, Brood No. 2.

| Number. | Amount of potassium hydrate in deci-normal solution, added to 200 c.c. of sea-water. | Amount of added alkali in the solution, expressed as a fraction of normal strength. | Observations of embryos at stated times after commencement of experiment. | | |
|---|---|---|---|---|---|
| | | | 4 h. 30 m. | 17 h. 30 m. | 24 h. 30 m. |
| 1 | Control | — | Two-celled stage ...................... | Morulæ and very early blastulæ (17 to 18 cells in optical section of circumference) | Blastulæ. Dead |
| 2 | 1 c.c. | 0·0005 | Two-celled, occasional three- or four-celled | Early blastulæ. More advanced than control | Blastulæ, and some early gastrulæ |
| 3 | 1·5 „ | 0·00075 | Two-celled, occasional three- or four-celled | Late blastulæ (26 cells in optical section of circumference). Best developed of series | Commencing gastrulæ. Dead |
| 4 | 2 „ | 0·0010 | Two-celled, some three- or four-celled | Blastulæ; but few developed ...... | Commencing gastrulæ. Dead |
| 5 | 3 „ | 0·0015 | Nearly all single-celled. A few two- or four-celled. Contents of cell granular and cells breaking up, accompanied in a few cases by irregular division | No morulæ. A few regular two-celled, and very irregular groups and cell divisions up to sixteen-celled. Many broken up, and free cells swimming about | No further development |
| 6 | 4 „ | 0·0020 | Very few cells visible; these are single except in one case, where there are two cells, one in process of division into three | All broken up, and gelatinous-looking masses of debris floating about | — |
| 7 | 5 „ | 0·0025 | Very few cells visible, none divided | Same as No. 6 | — |

## Table V.—Ammonium Hydrate.

Experiment 1, Brood No. 3.

| No. | Amount of ammonia in decinormal solution, added to 200 c.c. of sea-water. | Amount of added alkali in the solution, expressed as a fraction of normal strength. | Observations of embryos at stated times after commencement of experiment. | |
|---|---|---|---|---|
| | | | 2 hrs. | 16 h. 30 m. |
| 1 | Control | — | Division into two cells commencing | Blastulæ. Twenty to twenty-four cells in circumference |
| 2 | 0·5 c.c. | 0·00025 | Same as control ............. | Blastulæ. Twenty cells in circumference. A few morulæ |
| 3 | 0·75 „ | 0·00037 | Same as control .............. | Same as No. 2 |
| 4 | 1·0 „ | 0·0005 | Same as control .............. | Same as No. 2 |
| 5 | 1·5 „ | 0·00075 | Same as control .............. | Half morulæ and half blastulæ |
| 6 | 2·0 „ | 0·001 | No division ..................... | Same as No. 5 |

Experiment 2, Brood No. 6. In this series blastulæ formed with smaller number of cells in embryo.

| No. | Amount of ammonia in decinormal solution, added to 200 c.c. of sea-water. | Amount of added alkali in the solution, expressed as a fraction of normal strength. | Observations of embryos at stated times after commencement of experiment. | |
|---|---|---|---|---|
| | | | 18 h. 30 m. | 42 hrs. |
| 1 | Control | — | Four-celled, many morulæ and a few early blastulæ | All stages to early blastulæ |
| 2 | 1 c.c. | 0·0005 | Two-, three- and four-celled, and morulæ | Morulæ, at all stages |
| 3 | 2 „ | 0·001 | Many single-celled, and stages up to early morulæ | Early and late morulæ. Extrusion of cell contents from cells |
| 4 | 3 „ | 0·0015 | Nearly all single cells ......... | No further development. Extrusion of cell contents |

### Table VI.—Calcium Hydrate.

Experiment 1, Brood No. 5. The lime-water added required for neutralisation of 50 c.c., in one case 25·25 c.c. of N/10 HCl, and in a second trial 25·28 c.c.; this lies so near 25 c.c. that the solution was taken as N/20.

| No. | Amount of calcium hydrate in N/20 solution, added to 200 c.c. of sea-water. | Amount of added alkali in the solution, expressed as a fraction of normal strength. | Observations of embryos at stated times after commencement of experiment. | |
|---|---|---|---|---|
| | | | 18 hrs. | 42 hrs. |
| 1 | Control | — | Well-developed blastulæ...... | No further development. Commencing degeneration |
| 2 | 1 c.c. | 0·00025 | Blastulæ clearly more advanced than control, and showing more cell-division | Late blastulæ, one or two moving |
| 3 | 2 " | 0·0005 | Same as No. 2 .................. | Same as No. 2, but more in motion |
| 4 | 4 " | 0·001 | Well-developed blastulæ, but some deformed in shape | Blastulæ, but motionless |
| 5 | 6 " | 0·0015 | Morulæ, and occasional small blastulæ | Blastulæ, but not so far developed as No. 4 |
| 6 | 8 " | 0·002 | Nearly all single-celled; but occasional two-, three- or five-celled group | Nearly all single-celled. Those showing development are chiefly early irregular morulæ. Single cells show extended layer. |
| 7 | 10 " | 0.0025 | Single cells in many cases pear-shaped | No development; single cells showing extended layer |

A second experiment gave similar results.

## Experiments with Acids.

### Table VII.—Hydrochloric Acid.

### Experiment 1, Brood No. 1.

| No. | Amount of hydrochloric acid in N/10 solution, added to 200 c.c. of sea-water. | Amount of added acid in the solution, expressed as a fraction of normal strength. | Observations of embryos at stated times after commencement of experiment. | | | | |
|---|---|---|---|---|---|---|---|
| | | | 4 h. 30 m. | 18 h. 15 m. | 29 hrs. | 42 h. 30 m. | 70 hrs. |
| 1 | Control | — | Four-celled stage ... | Blastulæ. Motionless | Blastulæ, a few moving | Blastulæ, in rapid movement | Commencing gastrulæ. Motionless |
| 2 | 0·5 c.c. | 0·00025 | Same as control ... | Same as control ... | Same as control ... | Same as control ... | Blastulæ. Motionless |
| 3 | 1·0 ,, | 0·0005 | Same as control ... | Same as control ... | Same as control ... | Same as control ... | Same as No. 2 |
| 4 | 2·0 ,, | 0·0010 | Three-celled and four-celled | Half blastulæ and half morulæ | Not observed......... | Not observed......... | Same as No. 2 |
| 5 | 3·0 ,, | 0·0015 | Nearly all single-celled, a few two-celled | Morulæ ............... | Half blastulæ and half morulæ | Blastulæ. Some motionless | Same as No. 2, but few developed |
| 6 | 4·0 ,, | 0·0020 | Single-celled ......... | Four-, six-, and eight-celled | Six- and eight-celled. Irregular | Morulæ and half-formed morulæ. Some two- and four-celled | None past morula stage |
| 7 | 5·0 ,, | 0·0025 | Single-celled ......... | Single-celled* ...... | Single-celled ......... | Not observed......... | All single fertilised cells |

* At this period No. 7 was divided into two equal portions of 100 c.c., one being left unaltered, and the other having 2·5 c.c. of N/10 alkali added, in order to see if neutralisation of the acid would allow division to proceed. The result was negative, the cells having apparently been killed by the acid, although they remained well preserved until the end of the experiment.

Experiment 2, Brood No. 2. Gave a similar result, the limit at which cell-division ceased entirely to occur, lying at 4 c.c. of added N/10 hydrochloric acid in 200 c.c. of sea-water. For other experiments, see Combined Experiments.

Table VIII.—Acetic Acid.

Experiment 1, Brood No. 6.

| No. | Amount of acetic acid in N/10 solution, added to 200 c.c. of sea-water. | Amount of added acid in the solution, expressed as a fraction of normal strength. | Observations of embryos at stated times after commencement of experiment. | |
|---|---|---|---|---|
| | | | 18 h. 30 m. | 42 hrs. |
| 1 | Control | — | Many morulæ and early blastulæ. Some four-celled groups | All stages to well-formed blastulæ |
| 2 | 0·5 c.c. | 0·00025 | Morulæ, occasional early blastula. Not so far advanced as control | All stages to late morulæ. Division irregular |
| 3 | 1·0 „ | 0·0005 | Morulæ only. Irregular division | Same as No. 2 |
| 4 | 2·0 „ | 0·0010 | Early stages and irregular morulæ. Many cells not divided | Early morulæ, and some late morulæ. Cells breaking up |
| 5 | 3·0 „ | 0·0015 | None beyond six- and eight-celled stages | Chiefly early morulæ, and earlier stages. Irregular division in earlier stages |
| 6 | 4·0 „ | 0·002 | All single-celled ............... | No stages beyond eight-celled. Irregular division |
| 7 | 5·0 „ | 0·0025 | All single-celled ............... | All single-celled |

Table IX.—Carbonic Acid.

Experiment 1, Brood No. 4. A solution of carbonic acid in sea-water, obtained by bubbling $CO_2$ through, was prepared and added to the sea-water in the quantities stated. Titration of this solution with N/10 NaOH, and phenolphthaleïn as indicator, gave 100 c.c. = 9·5 c.c. N/10 alkali.

| Number. | Amount of carbonic acid in 0·0095 N solution added to 200 c.c. of sea-water, minus volume of carbonic acid sea-water added. | Amount of added carbonic acid in the solution, expressed as a fraction of normal strength. | Observations of embryos at stated times after commencement of experiment. | | |
|---|---|---|---|---|---|
| | | | 4 h. 45 m. | 19 hrs. | 42 hrs. |
| 1 | Control | — | Nearly all four-celled stage... | Chiefly morulæ, a few early blastulæ | Morulæ and blastulæ |
| 2 | 7 c.c. | 0·00033 | Two-celled, occasional four-celled. Irregular division | Smaller number of morulæ than in control. One or two commencing blastulæ | Morulæ. Less advanced than control |
| 3 | 14 „ | 0·00066 | Two-celled, occasional three-celled, no four-celled. Irregular division | Less advanced than No. 2. Fewer morulæ, and scarcely any of these perfect. Capsules gone | Broken-up, badly-developed morulæ in early stages |
| 4 | 21 „ | 0·00099 | Nearly all single-celled, occasional two-celled | Disintegrating. A few broken-up morulæ seen | All disintegrated |
| 5 | 28 „ | 0·00132 | Nearly all single cells ......... | No normal cell-division ............ | Degenerated cells |
| 6 | 35 „ | 0·00165 | All single cells .................. | One or two attempts at cell-division. Highest an irregular six-celled group | Same as No. 5 |
| 7 | Carbonic acid gas bubbled through | 0·0095 | All single cells .................. | No cell-division ..................... | No cell-division. The cells are well preserved, and fertilisation membranes are visible. |

For comparison with the other experiments, it may be pointed out that the amounts of carbonic acid added correspond approximately in cubic centimetres of N/10 solution to 0·7 c.c., 1·3 c.c., 2 c.c., 2·7 c.c., 3·3 c.c., and 19 c.c. So that the effect with carbonic acid in stopping growth occurs at about the same level as with hydrochloric or acetic acids. A second experiment with carbonic acid gave a similar result.

## EXPERIMENTS WITH ACID AND ALKALINE SALTS.

In preliminary experiments the range of concentration of each salt was determined at which the developing eggs were affected, as in the experiments with alkalies and acids recorded above, and afterwards a few concentrations were selected for comparison at about this range. In order to make the results comparable as between the different salts, and to gain a comparison also with the action of alkalies and acids, a combined series with the same brood of eggs was made including alkali, acid, and the alkaline and acid salts, two or three concentrations only being used of each substance.

Table X.

Experiment 1, Brood No. 8. In this experiment the fluids were changed each day by decantation and replaced by fluids made up of the same constitution and concentration.

| No. | Amount of chemical added, in c.c. of the gramme-molecular strength named, to 200 c.c. of sea-water. | Amount of added chemical in the solution, expressed as a fraction of gramme-molecular concentration per litre. | Observations of embryos at stated times after commencement of experiment. | | | | |
|---|---|---|---|---|---|---|---|
| | | | 5 h. 45 m. | 19 h. 45 m. | 31 hrs. | 43 hrs. | 50 h. 30 m. |
| 1 | Control | — | Two-, three-, and four-celled. Majority four-celled | Chiefly well-formed blastulæ, not in motion. Circumference 16—18 cells | Blastulæ, motionless | Blastulæ, motionless | Blastulæ, many in motion |
| 2 | 2 c.c. M/10 NaOH | 0·001 M | Two-, three- and occasional four-celled. Less advanced than control | Blastulæ. Clearly more advanced than control. Circumference 24—26 cells | Blastulæ, motionless | Degenerating...... | — |
| 3 | 3 c.c. M/10 NaOH | 0·0015 M | Chiefly two-celled, occasional three- or four-celled. Same as No. 2, but irregular division | Countable groups of four and over. Early and late morulæ and blastulæ. Less advanced than control | Blastulæ, motionless | Blastulæ, a few in motion | Blastulæ. Better preserved than control, and many in motion |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 4 | 2 c.c. M/10 HCl | 0·001 M | Single- and two-celled. More single than divided. Irregular division well-marked | No blastulæ. Very irregular morulæ at all stages of development. Often one-half only of egg developed | Degenerated ........ | — | — |
| 5 | 3 c.c. M/10 HCl | 0·0015 M | All single fertilised cells, many misshapen | No blastulæ. Scarcely any beyond early morulæ. Many abortive attempts at division | Degenerated ........ | — | — |
| 6 | 2 c.c. M/10 KOH | 0·001 M | Nearly all two-celled. Irregular four-celled and over. Cell-division markedly irregular | All stages to well-developed blastulæ. Many of the blastulæ are much further developed than in control (circumference 24 cells). Irregular division manifest in cells of earlier stages | Well-developed blastulæ. Better developed than any previous number in series | Blastulæ, many in active motion | Blastulæ. Better preserved than control. Large number in motion |
| 7 | 3 c.c. M/10 KOH | 0·0015 M | Many two-celled, also four-celled and irregular groups up to seven. Irregular division | None beyond early morula stage. Irregular division | Degenerated ........ | — | — |
| 8 | 3 c.c. M/10 $NaHCO_3$ | 0·0015 M | Two-, three-, four- and higher celled. Most advanced of series so far | All stages to well-developed blastulæ. More cells in circumference than in control. Irregular division | Degenerated ........ | — | — |
| 9 | 6 c.c. M/10 $NaHCO_3$ | 0·0030 M | Four-celled and higher irregular divisions. Well advanced | All stages to well-developed blastulæ, which are misshapen. Irregular division in earlier stages | Blastulæ, motionless | Degenerating...... | — |
| 10 | 10 c.c. M/10 $NaHCO_3$ | 0·0050 M | Four-celled and incomplete four. Not so far advanced as Nos. 8 and 9 | All stages to blastulæ, but a smaller number have passed morula stage. The blastulæ more advanced than in control | Blastulæ, motionless | Degenerating...... | — |

Table X—*continued.*

| No. | Amount of chemical added, in c.c. of the gramme-molecular strength named, to 200 c.c. of sea-water. | Amount of added chemical in the solution, expressed as a fraction of gramme-molecular concentration per litre. | Observations of embryos at stated times after commencement of experiment. | | | | |
|---|---|---|---|---|---|---|---|
| | | | 5 h. 45 m. | 19 h. 45 m. | 31 hrs. | 43 hrs. | 50 h. 30 m. |
| 11 | 3 c.c. M/10 $Na_2CO_3$ | 0·0015 M | Two-, three-, and four-celled, and irregular divisions beyond | All stages to well-developed blastulæ (circumference 24 cells). Marked irregular division | Blastulæ, several moving | Blastulæ, not in motion | Degenerating |
| 12 | 6 c.c. M/10 $Na_2CO_3$ | 0·0030 M | Same as No. 11. But more cells in higher stages | All stages to well-developed blastulæ | Blastulæ, motionless | Blastulæ, a few moving | Blastulæ, a few moving |
| 13 | 5 c.c. M/10 $NaH_2PO_4$ | 0·0025 M | All single fertilised cells. Misshapen | None beyond early morulæ. Irregular division in all stages | Degenerated ......... | — | — |
| 14 | 7 c.c. M/10 $NaH_2PO_4$ | 0·0035 M | Same as No. 13......... | All groups countable. Very few complete divisions beyond three-celled. Incomplete and irregular divisions | Degenerated ......... | — | — |
| 15 | 10 c.c. M/20 $Na_2HPO_4$ | 0·0025 M | Cell division irregular. Some groups up to sixteen cells | Morulæ and blastulæ at different stages. Majority regular and well-formed | Morulæ and blastulæ, degenerating | — | — |
| 16 | 20 c.c. $Na_2HPO_4$ | 0·0050 M | Two- and four-celled about equal | All stages to early blastulæ | Morulæ and blastulæ, degenerating | — | — |
| 17 | 30 c.c. $Na_2HPO_4$ | 0·0075 M | Chiefly four-celled and some irregular divisions beyond | Morulæ only .................. | Morulæ, degenerating | — | — |

Table XI.

Experiment 2, Brood No. 9. The embryos developed in this experiment were fixed, sectioned, and stained, as described and the preparations were used for making the illustrations given (figs. 1 to 17 and 19 to 30).

| No. | Amount of chemical added, in cubic centimetres of the gramme-molecular strength named, to 200 c.c. of sea-water. | Amount of added chemical in the solution, expressed as a fraction of gramme-molecular concentration per litre. | Observations of embryos at stated times after commencement of experiment. | |
|---|---|---|---|---|
| | | | 3 h. 45 m. | 8 hrs. |
| 1 | Control | — | Nearly all two-celled. Occasional incomplete three- or four-celled. Very regular division | Early morulæ |
| 2 | 2 c.c.<br>M/10 NaOH | 0·001 M | More advanced than control. More three- and four-celled; occasional higher division. Tendency to irregular division | Morulæ. More advanced than control |
| 3 | 3 c.c.<br>M/10 NaOH | 0·0015 M | Very irregular division. Two-, three-, and four-celled, and more advanced groups with cells of irregular shape and size | Two-celled, three-celled, and irregular groups of eight cells or more. Very irregular division. Less advanced than control |
| 4 | 2 c.c.<br>M/10 HCl | 0·001 M | Chiefly two-celled. Some incompletely divided, regular and irregular in size. Irregular and incomplete four- and eight-celled groups | Two-, three-, and four-celled groups, and early morulæ. Irregular division |
| 5 | 3 c.c.<br>M/10 HCl | 0·0015 M | Practically all single-celled. One or two incomplete two-celled | Nearly all single-celled. Occasional incomplete and complete two-celled, irregular three celled; none further advanced |
| 6 | 2 c.c.<br>M/10 KOH | 0·001 M | Nearly all complete and regular two-celled. A few three- and four-celled | Morulæ fairly advanced; some irregular. More advanced than control |
| 7 | 3 c.c.<br>M/10 KOH | 0·0015 M | Excessively irregular divisions. More advanced in division than control. All stages, from complete and irregular two-celled to early morulæ, with irregular-sized cells | Very irregular two-, three-, and four-celled, and early morulæ. Less advanced than control |
| 8 | 3 c.c.<br>M/10 NaHCO$_3$ | 0·0015 M | Nearly all regular and complete four-celled. Occasional eight- and sixteen-celled group. Obviously more advanced than control, examined alongside, and now partially in four-celled stage | Advanced morulæ. Fairly regular |

Table XI—*continued.*

| No. | Amount of chemical added, in cubic centimetres of the gramme-molecular strength named, to 200 c.c. of sea-water. | Amount of added chemical in the solution, expressed as a fraction of gramme-molecular concentration per litre. | Observations of embryos at stated times after commencement of experiment. | |
|---|---|---|---|---|
| | | | 3 h. 45 m. | 8 hrs. |
| 9 | 6 c.c. M/10 $NaHCO_3$ | 0·0030 M | Nearly all complete four-celled ...................... | Morulæ. Slightly less advanced than No. 8 |
| 10 | 10 c.c. M/10 $NaHCO_3$ | 0·0050 M | Nearly all four-celled; a few six- and eight-celled. Occasional irregularity in size of cells | Morulæ. Same as Nos. 8 and 9, but slightly irregular |
| 11 | 3 c.c. M/10 $Na_2CO_3$ | 0·0015 M | Nearly all regular four-celled. A few irregular, and with larger number of cells | Morulæ. Slightly more advanced, and more regular than No. 10 |
| 12 | 6 c.c. M/10 $Na_2CO_3$ | 0·0030 M | Many regular four-celled, but several two- and three-celled; also early morulæ, with irregular shape of group and irregular size of cell | Morulæ. Less advanced than No. 11 |
| 13 | 5 c.c. M/10 $NaH_2PO_4$ | 0·0025 M | Practically all single celled. Occasional irregular and incomplete three-celled | Very irregular divisions into two- and three-celled, but majority single-celled. No morulæ |
| 14 | 7 c.c. M/10 $NaH_2PO_4$ | 0·0035 M | All single-celled ...................................... | Very irregular attempts at division into two- and three-celled, but practically all single-celled |
| 15 | 10 c.c. M/20 $Na_2HPO_4$ | 0·0025 M | Complete and incomplete four-celled. Irregular, but not so much so as Nos. 3 and 7 | Fairly advanced morulæ |
| 16 | 20 c.c. M/20 $Na_2HPO_4$ | 0·0050 M | Chiefly in four-celled groups, but many containing more cells; also two- and three-celled groups. Irregularity in size and arrangement of cells more marked than in No. 15 | More single cells, and morulæ where they have developed, much less advanced than in No. 15. Also more irregular cell-division |
| 17 | 30 c.c. M/20 $Na_2HPO_4$ | 0·0075 M | Majority four-celled, but also two-, three-, six-, and eight-celled groups. Irregular division, well marked. | Similar to No. 16, but somewhat less advanced in division, and cells more irregular in size |

### *Summary of Results on Rapidity of Growth.*

The following conclusions may be drawn from the observations on the developing eggs in the living condition recorded in Tables I to XI, which are also illustrated by figs. 1 to 17, drawn from sections of the eggs developed in the experiment described in Table XI. The sections were drawn under a low magnification (61 diam.) with the Zeiss Zeichen-ocular. The eggs shown in the drawings had all developed, for the same period (8 hours) in the different media specified, from the same brood of eggs, and the eggs were subsequently fixed and stained as described above.

1. The extreme limits of variation of hydrogen and hydroxyl ion within which growth is possible are very narrow. Addition of 0·0015 M. of caustic alkali (see figs. 3 and 7) on the one hand or of 0·001 M. of acid (see fig. 4) practically stopping all cell-division.

2. A slight increase in alkalinity favours growth and cell-division and at the same time tends to produce irregularity in size and shape of the resulting cells (contrast fig. 1 with figs. 2, 6, 8, 9, 10, 11, 12, 15, 16, 18).

3. In the case of small additions of acids no such favouring action is observable, but from the beginning cell-division and growth are inhibited (see Tables).

4. Increase in alkali above the optimum amount leads to increased and irregular nuclear division unaccompanied by complete cell-division. As a result the cells become multi-nucleated. The cells also become excessively irregular in size and shape (see figs. 3, 7, 8, 10, 15, 16, 17, 18).

5. On the other hand increased acidity leads in many cases to action upon the chromatin of the nuclei, so that in the sections the nuclei stain faintly and are comparatively few in number, and there is no proliferation of nuclei in the undivided cells, similar to that seen in the case of alkali (*see* figs. 4, 5, 13, 14).

6. The primary factors affecting the rate of growth appear to be the variations in concentration of hydroxyl and hydrogen ions. Thus all the caustic alkalies are of approximately equal power and there is little or no action of the Kation (see Tables and contrast figs. 2 and 3 with figs. 6 and 7). But in the case of the phosphates of the alkalies where the hydrogen and hydroxyl ion concentrations are comparatively low, there appears in addition to be a specific factor. See the marked action of 0·0025 M. $NaH_2PO_4$ in practically stopping cell division, while 0·0050 M. of $Na_2HPO_4$ has a favouring action (contrast figs. 13 and 14 with figs. 15, 16, 17).

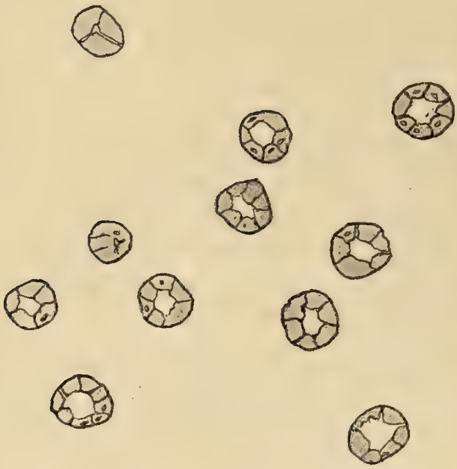

FIG. 1.—Control. Growth of eggs in normal sea-water.

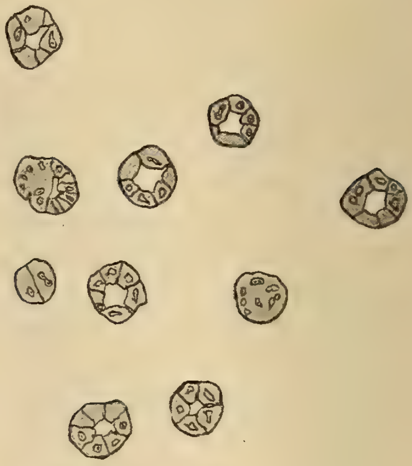

FIG. 2.—Growth of eggs in sea-water, +0·001 M. sodium hydrate.

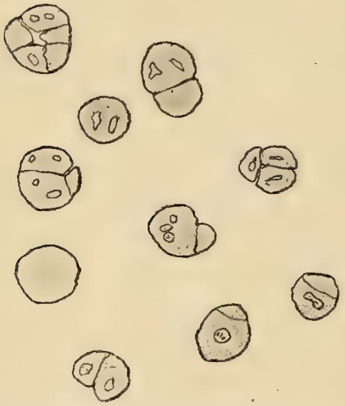

FIG. 3.—Growth of eggs in sea-water, +0·0015 M. sodium hydrate.

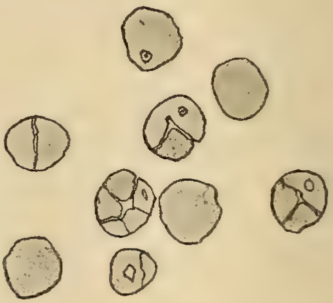

FIG. 4.—Growth of eggs in sea-water, +0·001 M. hydrochloric acid.

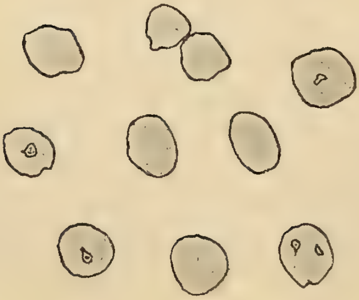

FIG. 5.—Growth of eggs in sea-water, +0·0015 M. hydrochloric acid.

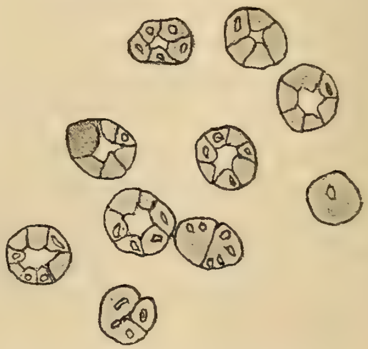

FIG. 6.—Growth of eggs in sea-water, +0·001 M. potassium hydrate.

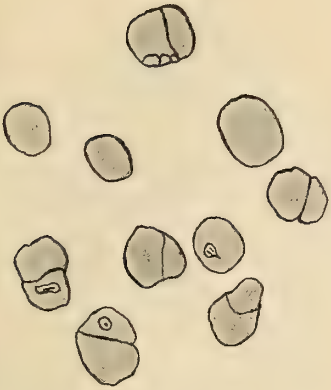

FIG. 7.—Growth of eggs in sea-water, +0·0015 M. potassium hydrate.

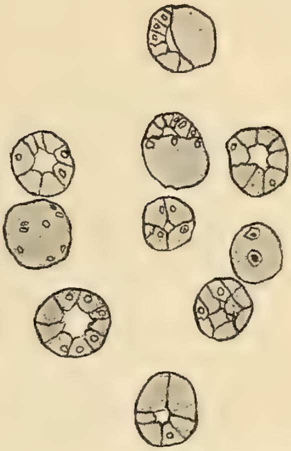

FIG. 8.—Growth of eggs in sea-water, +0·0015 M. sodium bicarbonate.

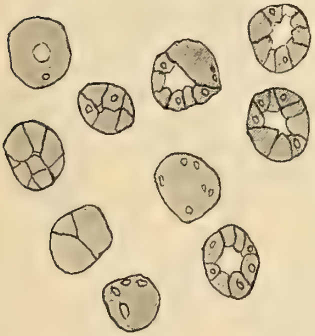

FIG. 9.—Growth of eggs in sea-water, +0·003 M. sodium bicarbonate.

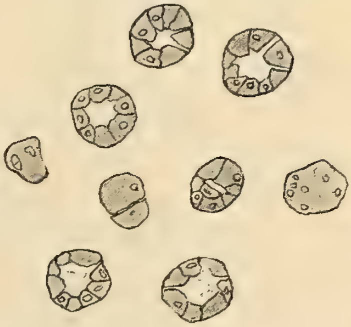

FIG. 10.—Growth of eggs in sea-water, +0·005 M. sodium bicarbonate.

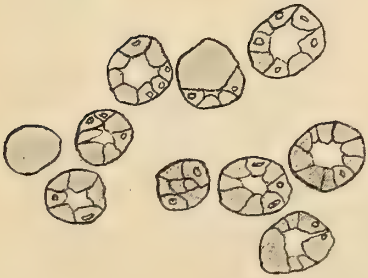

FIG. 11.—Growth of eggs in sea-water, +0·0015 M. sodium carbonate.

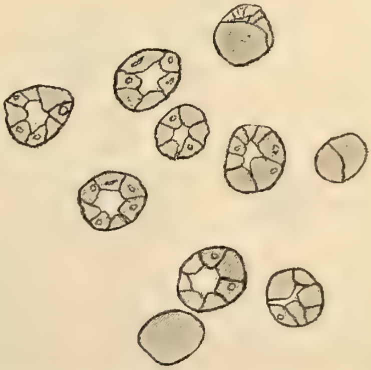

FIG. 12.—Growth of eggs in sea-water, +0·003 M. sodium carbonate.

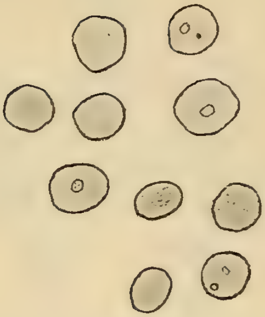

Fig. 13.—Growth of eggs in sea-water, +0·0025 M. mono-sodium phosphate.

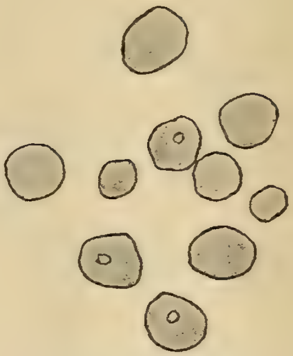

Fig. 14.—Growth of eggs in sea-water, +0·0035 M. mono-sodium phosphate.

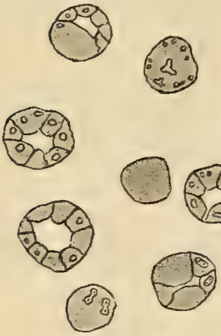

Fig. 15.—Growth of eggs in sea-water, +0·0025 M. di-sodium phosphate.

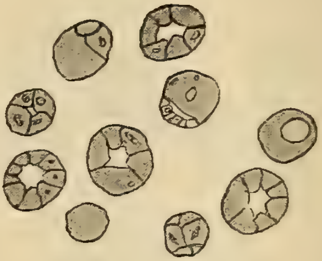

Fig. 16.—Growth of eggs in sea-water, +0·005 M. di-sodium phosphate.

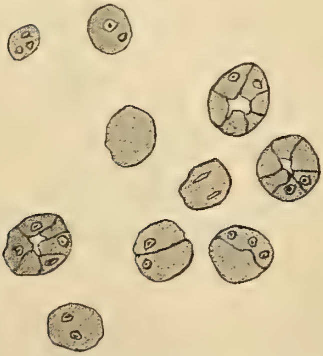

Fig. 17.—Growth of eggs in sea-water, +0·0075 M. di-sodium phosphate.

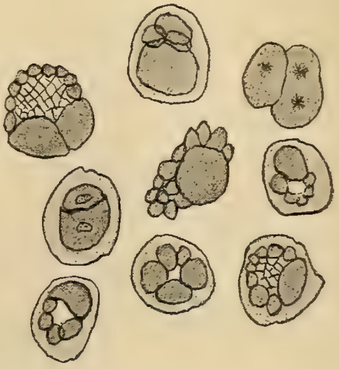

Fig. 18.—Drawings of cells in fresh condition developed in alkaline media, showing irregularities in size and shape of cells.

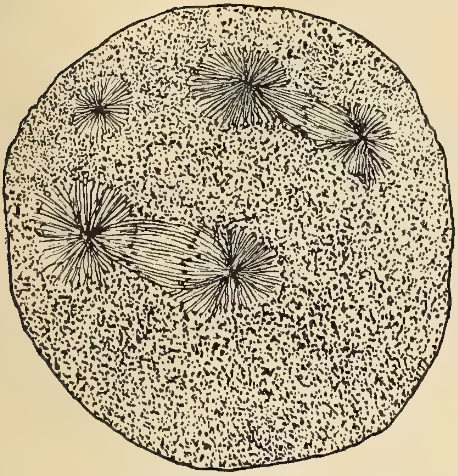

Fig. 19.—Undivided egg in sea-water, +0·0025 M. di-sodium phosphate, showing two dividing nuclei.

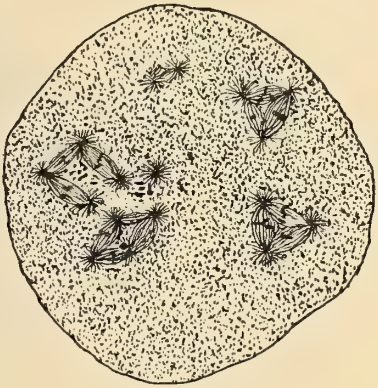

Fig. 20.—Undivided egg in sea-water, +0·001 M. potassium hydrate, showing several multipolar mitoses.

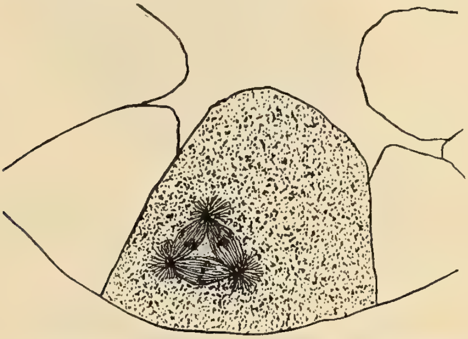

Fig. 21.—Cell in blastula in sea-water, +0·001 M. potassium hydrate, showing multipolar mitosis.

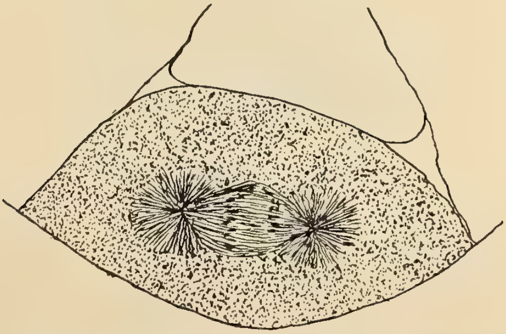

Fig. 22.—Cell in morula in sea-water, +0·001 M. potassium hydrate, showing asymmetrical mitosis—unequal number of chromosomes.

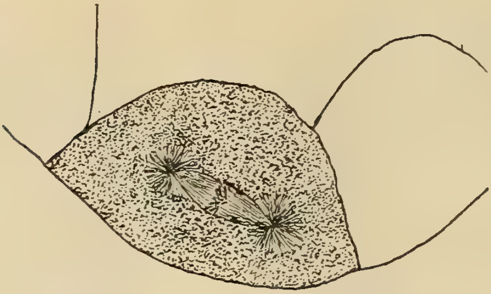

Fig. 23.—Cell in blastula in normal sea-water.

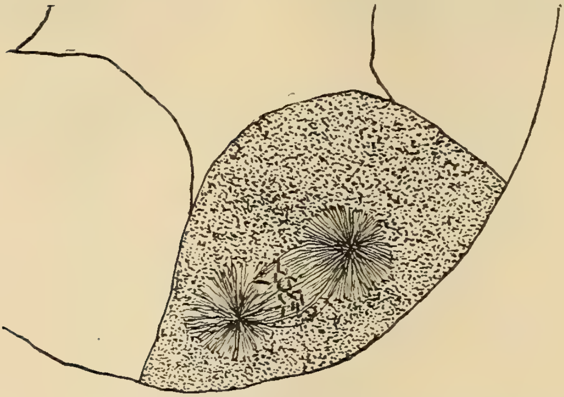

Fig. 24.—Cell in blastula, in sea-water, +0·0025 M. di-sodium phosphate, showing rod chromosomes, unreduced in number

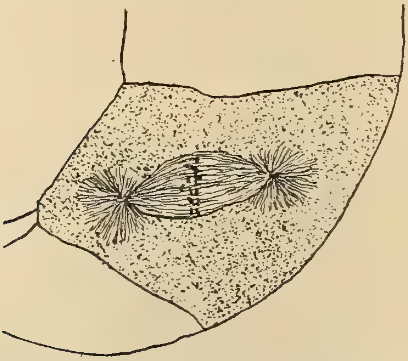

Fig. 25.—Cell in morula, in sea-water, +0·005 M. di-sodium phosphate, showing partial shortening of rod chromosomes.

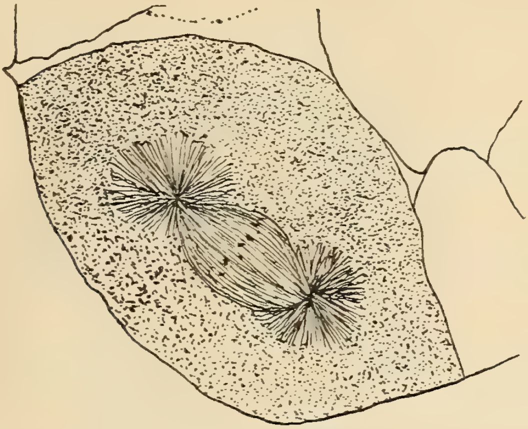

FIG. 26.—Cell in morula, in sea-water, +0·0075 M. di-sodium phosphate, showing shortening of rod chromosomes to dots, and reduction in number.

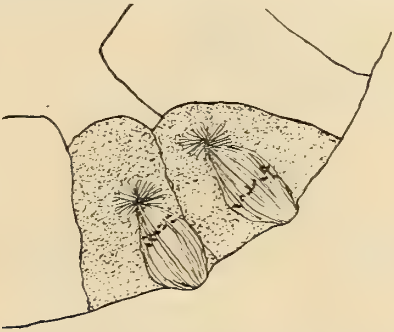

FIG. 27.—Two cells in blastula, in sea-water, +0·005 M. di-sodium phosphate, showing partial shortening and reduction of chromosomes and protrusion of spindles, as if polar bodies were being formed.

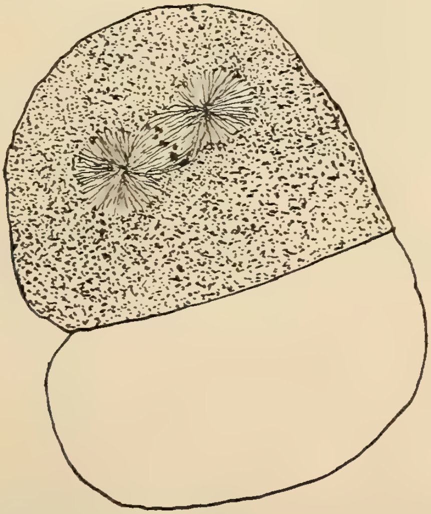

FIG. 28.—Egg in two-celled stage in sea-water, +0·0015 M. potassium hydrate, showing shortening of dividing chromosomes to dots and reduction in number.

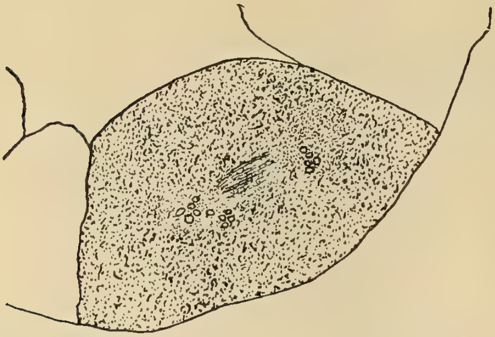

Fig. 29.—Cell in blastula, in sea-water, +0·0025 M. di-sodium phosphate. (See text, p. 134.)

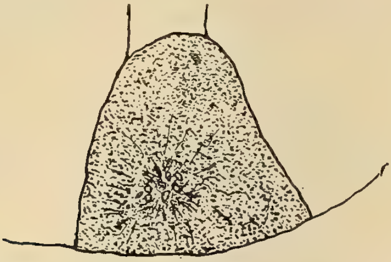

Fig. 30.—Cell in blastula in 0·005 M. di-sodium phosphate. (See text, p. 134.)

### *Pathological Nuclear Divisions.*

The irregularity of growth in sea-water to which alkalies or alkaline salts have been added is accompanied by characteristic irregularities in the mitotic divisions, which are illustrated in figs. 19 to 30.*

*Multiple Nuclei.*—The divisions in the cytoplasm occur less frequently than in the nuclei, so that the cells become multi-nucleated (see figs. 2, 6, 8, 9, 10, 15, 19, 20). This occurs both in undivided and divided eggs. In many cases two or more such active dividing nuclei are found in the same cell (fig. 19).

*Multipolar Mitosis.*—This occurs both in single-celled eggs and in the later stages (see figs. 20 and 21). The chromosomes are unequally distributed to the different spindles.

*Asymmetrical Mitosis.*—This frequently occurs in the various alkaline solutions, and gives rise to unequal nuclei. The cause of the unequal number of chromosomes appears to be that some of the chromosomes are carried to one pole without previous division. (See fig. 22, and lower spindle in fig. 19.)

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* The figs. 19 to 30 were drawn with the Zeiss Zeichen-ocular, the finer details being filled in as realistically as possible, under a Leitz $\frac{1}{12}$ oil immersion objective. The magnification measured by the stage micrometer was 790 diameters.

*Changes in Chromosomes and Reduction in Number.*—The short, rod-shaped chromosomes of the organism which we have been examining (*Echinus esculentus*) do not show typically in its normal maturation or maiotic divisions the appearances described by Farmer and Moore* and others in maiotic division, and by Farmer, Moore, and Walker† in cancer.

According to Bryce,‡ the main changes in *Echinus esculentus* during maturation consist in a marked shortening and thickening of the chromosomes with reduction in number. Each chromosome divides into two short curved rods with spherical enlarged ends, giving rise to an appearance similar to tetrads. But at no time are there any rings or other irregular figures, as described in so many other organisms, and there is no true tetrad formation.

R. Hertwig§ has observed in the case of Echinus a formation of bodies described as resembling tetrads, as a result of addition of dilute poisons.

In our specimens of eggs grown in dilute alkali, we have observed appearances in many cases similar to those shown in the drawings illustrating Bryce's paper.

As the amount of added alkali is increased, there occurs both a shortening and thickening of the chromatin rods and a reduction in their number. In some cases the appearance of rods is lost entirely, and the chromatin becomes arranged in minute masses resembling tetrads. These changes are illustrated in figs. 24, 25, 26, 27, and 28. Thus in the three strengths of di-sodium phosphate solution a shortening of the chromosomes in fig. 25 is seen as compared with fig. 24, and in the strongest solution, fig. 26, the rods are reduced to dots and the number is decreased to approximately one-half. In fig. 28 some of the chromosomes present a tetrad-like appearance. These changes, while frequent in occurrence, are not seen in all the dividing cells in any strength of solution, but occur in increased number in the stronger solution. A peculiar appearance is represented in fig. 27, in which a decided protrusion of one pole of the spindle in two adjacent cells of a blastula was observed beyond the cell margin, as if a polar body were being formed.

Although it is difficult to count the chromosomes accurately, the reduction in number is obvious on contrasting the weaker and stronger solutions.

In a fair number of cells, especially in the di-sodium phosphate solutions, a peculiar arrangement of the chromatin is observed, which is illustrated in fig. 29. The entire chromatin of the cell is seen to be arranged in two groups of circles at a distance apart corresponding to the usual distance of the centrosomes of a spindle, and in some cases, as in fig. 29, the remains of achromatic fibres are indistinctly seen between the two groups of circles. The cytoplasm surrounding each group of circles is distinctly lighter and freer from granules than the rest of the cytoplasm. In structure each circle consists of a thin ring of chromatin showing distinct thickenings, about four in number, arranged approximately about equidistant around the circumference. The interior of the circle is clear, and both the outer and inner border are clearly marked. In some cases this arrangement of the chromatin in circles is also seen where there is only one group of circles as in fig. 30.

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* 'Q. J. M. S.,' vol. 48, 1905, p. 489.

† 'Roy. Soc. Proc.,' vol. 72, 1903, p. 499.

‡ 'Q. J. M. S.,' vol. 46, 1903, p. 177.

§ 'Sitz. Ber. Ges. Morph. und Phy.,' München, 1895. Quoted from Wilson, "The Cell in Development and Inheritance," 1904, p. 256.

### *Discussion of Results and Summary.*

Our attention was attracted to the study of the effects of small variations in reaction upon the growth of cells from the bio-chemical point of view, as a result of the observation that in malignant disease no hydrochloric acid is in general secreted by the gastric glands, no matter where the malignant growth is situated, which pointed to an increased alkalinity of the plasma.

In the course of our investigations upon the rate of growth of the cell, when microscopic examination was made of the cells in the fresh condition, we were struck by the marked irregularities in size and shape of the developing cells in the alkaline media illustrated in fig. 18, which is drawn from cells in the fresh solution developing in sea-water, to which di-sodium phosphate has been added, and also by the marked tendencies to nuclear proliferation.

This led us secondarily to a cytological investigation of the cells when fixed and stained to show nuclear division, as a result of which we have found the irregular forms of mitosis described in the text. These atypical divisions, which have been produced by variations in the medium similar to those which occur in the blood in cases of malignant disease, closely resemble the pathological divisions seen in the growths of malignant disease.

The results of our experiments and their relationship to the processes in malignant growths may be summarised as follows:—

1. In nearly all cases of malignant disease the secretion of hydrochloric acid by the gastric glands is stopped or greatly reduced, and this effect is not due to local conditions in the stomach, since it occurs wherever the growth is situated; but is due to a change in the distribution of salts in the plasma whereby the alkalinity is increased or the concentration in hydrogen ions diminished.

2. Addition of *small* amounts of alkalies or alkaline salts, such as di-sodium phosphate, to the medium in which cells are growing and dividing,

causes at first an increase in rate of growth and division, but as the amount is increased, there appears a marked tendency to irregularity in size and shape of the resulting cells. Nuclear division becomes in advance of cytoplasmic division, so that the cells become multi-nucleated. As the alkali is further increased, both cell division and nuclear division are stopped.

3. Accompaning the increased stimulus to nuclear division given by the dilute alkali, there are seen many of the atypical forms of mitosis described in malignant growths. The variations from the normal illustrated in the drawings are: (1) multiple nuclei in the same cell in active division; (2) multipolar mitosis, occurring both in the single cell stage, and later in the development of the organism; (3) asymmetrical mitosis, leading to unequal distribution of chromosomes to the two daughter cells; (4) reduction in length of the chromosomes as the strength of alkali is increased until the chromosomes appear as rounded dots, and accompanying the reduction in length there is also a reduction in number to about one-half the normal; (5) in certain cases the chromatin becomes arranged in circles, each of which shows a number of thickenings. The circles are arranged in groups in the cell, and appear to represent a stage in the anaphase, the groups being placed at about the usual distance apart of the centrosomes, and traces of the achromatic fibres being occasionally visible.

4. No such increased growth or stimulus to nuclear division is given by varying the normal reaction of the medium in the opposite direction, by the addition of equal small amounts of acid. From the beginning the minutest amount of added acid has an inhibitory effect upon growth and nuclear division. There is no nuclear proliferation as the amount of acid is increased, and at a very slight amount of increased acidity all attempt at cell-division ceases. In the fixed and nuclear stained preparations cell-division figures are absent, and in the resting nuclei the staining power of the chromatin is decreased, so that the nuclei present a washed-out appearance contrasted with the normal nuclei or those of organisms grown in dilute alkaline solution.

5. The extreme limits at which life and cell-division are possible lie close together, indicating that the cell is very sensitive to even slight changes in the concentration of hydrogen and hydroxyl ion concentration. Thus the addition of so little as 0·0015 M. of either alkali or acid to sea-water practically stops all growth. On account of the presence of phosphates and carbonates in the sea-water the change in hydrogen and hydroxyl ions caused by such additions cannot be large.

[*Note added October* 24.—Since the paper was written we have had an opportunity through the kindness of Messrs. J. E. S. Moore and C. E Walker of the Liverpool Cancer Research, of examining the appearances represented in figs. 29 and 30 under a specially high magnification of about 3000 diameters.

The magnification was obtained with a 10-inch tube, 3 mm. Zeiss apochromatic objective, 27 compensating ocular, and with monochromatic green illumination. Messrs. Moore and Walker have pointed out to us that these bodies, which appear as described in the text when seen with an ordinary $\frac{1}{12}$-inch oil immersion, are, when seen with the 3000 diameter magnification, really spheroidal bodies consisting of an inner mass or vesicle which scarcely stains at all, over which ramify filaments of deeply staining chromatin. Further examination with this magnification also demonstrates that the chromosomes represented as dots in fig. 28 are, in reality, also vesicular chromosomes. Even with the $\frac{1}{12}$-inch oil immersion we had been able to see a well-marked black line around the periphery of each dot, but had been unable to determine whether or not this was merely an optical effect. The 3000 diameter magnification, however, clearly shows that the structure is the same as that of the larger masses in figs. 29 and 30.

It is interesting that these minute chromosome masses occur upon the spindle in exactly similar fashion to the normal rod-shaped chromosomes, and that wherever they occur there is a diminution in the number of chromosomes. In many cases an occurrence of both rod chromosomes and spheroidal chromosomes is observable upon the same spindle as if conversion had only partly taken effect.

Where conversion of the entire number of chromosomes into the spheroidal variety is seen upon the spindle, the number of such chromosomes is usually approximately half the normal number, as in fig. 28. But when they are found after separation has taken place, as in figs. 29 and 30, the number is very variable, and as the number diminishes there is a corresponding increase in size in the individual chromatic vesicles.

We are informed by Messrs. Moore and Walker that bodies presenting a similar appearance are seen in malignant growths, and also under other pathological conditions, and in certain normal tissues.

The question of the relationship, if any, of the diminution in number when such spheroidal chromosomes are formed, to the reductions occurring in normal maiotic division, and in cancer cells, we prefer to leave at the present entirely open, since we have no evidence that the diminution in number is effected by the same means; and we merely point out the diminution of the number of chromosomes upon the spindle as a result of increasing the alkalinity of the medium, as an interesting and suggestive fact.]

# *A Note on the Effect of Acid, Alkali, and certain Indicators in Arresting or otherwise Influencing the Development of the Eggs of* Pleuronectes platessa *and* Echinus esculentus.

By Edward Whitley, M.A. (Oxon.).

(Communicated by Professor W. A. Herdman, F.R.S. Received November 14,
—Read November 23, 1905.)

## I. Effect of Acid and Alkali on the Eggs of Pleuronectes.

While working last spring at the Port Erin Biological Station on the effect of acids and alkalies upon the development of Echinus eggs, it was suggested to me that it might be interesting to try the general effect of similar solutions upon some other type of organism.

For this purpose the eggs of the Plaice (*Pleuronectes platessa*) were selected, as they were to be obtained in abundance from the fish-hatchery attached to the station, but, as time pressed, it was only found possible to experiment with one acid and one alkali, and decinormal solutions of hydrochloric acid and sodium hydrate were accordingly used.

In a pond attached to the hatchery numbers of plaice are kept in the spawning season, and the surface of the pond is skimmed each morning for the purpose of collecting the eggs, which are then placed in the hatching apparatus. Three batches of these eggs of different ages were taken for experimentation—those freshly skimmed from the pond, those which had been removed two days before and had remained since in the hatching apparatus, and those taken 10 days before and similarly treated. Some few eggs always escape the net in the process of skimming, and are taken in the catch of later days, so that, when 10 days old eggs, for instance, are spoken of, what is meant is that none can be younger than that, although some few may be slightly older. Such as showed obvious differences in age from the majority of each batch were removed.

The eggs were treated in a similar way to that employed in a research carried out at the same time on Echinus eggs,* namely, they were placed in small batches in a number of tumblers, each containing 200 c.c. of sea-water, to which measured amounts of the decinormal solutions of acid or alkali were added. A summary of previous work on the effects of acid and alkaline solutions upon development will be found in the paper above referred to.

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* See preceding paper.

The accelerating effect of small quantities of alkali on growth observed by Loeb in the case of Tubularia,* and the eggs of Arbacia,† and in those of Echinus, in the research mentioned above, was not noticed, but attention may be drawn to the very powerful results of even a small disturbance of the chemical equilibrium, which can be seen from Table I, where it will be observed that, after six days, 4 c.c. of decinormal acid or alkali in 200 c.c. of sea-water (*i.e.*, a five-hundredth normal solution) produces a death-rate among fresh eggs of 75 and 44 per cent. respectively, against only 5 per cent. in the Control.

In the experiments above alluded to with the eggs of *Echinus esculentus*, it was found that acids and acid salts above a very small concentration produced more deadly effects than corresponding quantities of alkalies and alkaline salts, and this was also found to be the case with Pleuronectes, and can be well seen in the same table (I) for strengths of 2·5 c.c. and upwards, especially with the younger eggs.

Probably three factors enter into the explanation:—

(1) The fact that part of the alkali added is immediately thrown out of solution as insoluble hydrates or carbonates.

(2) Alkali is constantly being used up to neutralise the acid products of metabolism—chiefly $CO_2$.

(3) According to Loeb, the presence of weak alkali assists the absorption of oxygen by the organism. If this be the case, the eggs in the acid solutions not being able to absorb oxygen so readily as the others, might probably be less resistant to the action of the reagent.

The tables seem also to show conclusively that the younger eggs are far more sensitive to the action of the acid or alkali, and generally to the influences of their environment than are the older eggs, or newly-hatched larvæ, which are, indeed, extraordinarily resistant. Thus, on referring again to Table I, it will be seen that a very large percentage of the older eggs survived 7, and even 11, days' treatment, whereas, of the fresh eggs, over 25 per cent. in the Control, and a much larger number in all the other cases were dead within 10 days. In this table the effect only of very small quantities of acid or alkali is given (five-hundredth normal and under), but experimentation with somewhat larger amounts gave the same result as shown in Table II. Thus, it needed only 6 c.c. of decinormal sodium hydrate to kill all the fresh eggs in four days, but more than 8 c.c. to kill all those of two days old in the same time, while, by the end of that period, 30 c.c. of alkali which was the greatest strength employed, had killed only 65 per cent. of the 10-days-old eggs. Acids, above a small concentration, had a remarkably stronger effect—in one day even 10-days-old eggs being killed by 10 c.c., and the two other batches by 6 c.c.

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* 'Univ. of California Publications, Physiol.,' vol. 1, 1904, p. 137, and 'Arch. f. gesant. Physiol.,' vol. 101, 1904, p. 340.

† 'Arch. f. Entwickelungsmechanik,' vol. 7, p. 631.

When working with the larger quantities of alkalies, it was very difficult to tell exactly when an egg was dead. In the first experiments the point was taken at which the egg began to become opaque, but, as soon as the percentage of alkali present exceeds a very small amount, precipitation of calcium and magnesium hydrates takes place, which renders observation of such a change in the transparency of the egg difficult. Loeb, in his experiments on Fundulus,* finds that the precipitate itself acts injuriously upon the eggs, and that, if this be filtered off, the eggs will live and develop in much stronger solutions than they would otherwise do. The process of filtering off the precipitate before placing the eggs in the solutions was not tried in the course of these experiments, they having been carried out previously to the reading of Loeb's paper.

Hitherto, it has always been considered that the young larva, on first hatching from the egg, enters upon the most critical stages in its career, and is at that time most susceptible to external influences, but the experiments here carried out appear to show a resistance steadily increasing with age, and that, even after the rupture of the egg-capsule, the young larva is, at all events, no more susceptible than just before that event. It may be suggested that this steadily increasing resistance is due to the gradual development of the epidermal cells, which would form a protection to the young embryo more or less impervious to the surrounding solution.

Tables III, IV, and V give the actual experimental data on which Table I is founded, and of which it is a *résumé*. Table VI is introduced with the object of laying emphasis on the statement as to the resisting powers of the older eggs. It shows the percentage of these eggs which succeeded in hatching in spite of the very unnatural conditions (stagnant water, possible overcrowding, etc.) in which they were placed. Incidentally may be noticed the very deadly effect of "di-methyl," to which attention is now to be drawn.

## II. Effect of Indicators on Pleuronectes and Echinus.

The effects which di-methyl-amido-azo-benzol and phenol-phthalein produce upon living organisms, as illustrated by the eggs of Pleuronectes and *Echinus esculentus*, were accidentally discovered in the course of these experiments. These indicators were originally added to the contents of some of the tumblers, to show any changes in reaction that might take place during growth. When it was observed that they had a specific action upon the eggs, a series of experiments was undertaken with them, the results of which are shown in Tables VII—X. In all the experiments, except those recorded in Table X, two drops of the indicator were added to 200 c.c. of sea-water, or sea-water plus varying quantities of alkali, in a tumbler. Table X records the results of varying the amount of phenol-phthalein employed. It will be seen that, although the indicators were made up in alcoholic solution, the amount of alcohol added in each case to 200 c.c. of liquid, was quite insufficient to materially influence the result.

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* 'Arch. f. Entwickelungsmechanik,' vol. 7, p. 631.

The experiments without indicators, recorded in Tables VIII and IX, are included for purposes of comparison.

It will be observed from the figures obtained that dimethyl is very deadly to the eggs of Pleuronectes and phenol-phthalein innocuous, while the opposite holds good with Echinus, the dimethyl having, if anything, a favouring effect on growth, and the phenol-phthalein being very injurious. Before killing, phenol-phthalein appears to be very effective in producing irregular divisions. The dimethyl was readily absorbed as such by both the organisms, staining them a deep yellow, so there can be no question as to its having thoroughly penetrated the tissues.

So far as can be ascertained, this specific action of indicators has not been noted before, and no explanation can be given of the fact of the different indicators affecting the two organisms in exactly opposite ways. Tadpoles in tap-water, to which the same, and even much larger amounts of these indicators had been added, appeared to be totally unaffected by either.

It might be of interest to repeat the experiments with other organisms, and with other organic compounds not known already to act as poisons.

## *Summary.*

(1) The amount of variation from the normal concentration of hydrogen and hydroxyl ions in sea-water which the eggs of Pleuronectes will tolerate is very small.

(2) A disturbance of the equilibrium towards the acid side is much more fatal than the opposite.

(3) A progressive development of resistance to an unfavourable action of the environment takes place in proportion to the age of the eggs.

(4) Phenol-phthalein is deadly to the eggs of *Echinus esculentus*, but harmless to those of Pleuronectes, while dimethyl quickly kills the latter, and appears, if anything, to have a favourable influence upon the development of the former.

My best thanks, in conclusion, are due to Professor Herdman, F.R.S., to whose kindness I am indebted for the material for these experiments, and for permission to work at the station, and to Professor Moore, for his kind and valuable criticism and assistance throughout.

Table I.—Comparison of Percentages of Deaths in Fresh Eggs. Eggs of 2 days old and eggs of 10 days old, with varying quantities of decinormal NaOH and HCl.

| | After 6 days. Fresh eggs. | After 7 days. 2 days old. | After 7 days. 10 days old. | After 10 days. Fresh eggs. | After 11 days. 2 days old. | After 11 days. 10 days old. |
|---|---|---|---|---|---|---|
| Control, 200 c.c. sea-water. | 5·0 | 0 | 2·0 | 25·0 | 0 | 2·4<br>(9 days) |
| 1·0 c.c. decinormal NaOH | 4·3 | 0 | 4·2 | | | |
| 1·5 „ „ | 9·6 | 0 | 2·9 | | | |
| 2·0 „ „ | 25·0 | 0 | 0·9 | | | |
| 2·5 „ „ | 18·2 | 2·6 | 3·0 | 31·8 | 2·6 | |
| 3·0 „ „ | 27·3 | 12·7 | 2·5 | 31·8 | 14·5 | 2·5 |
| 4·0 „ „ | 44·0 | 25·0 | 9·4 | 48·0 | 48·2 | 26·9<br>(10 days) |
| 1·0 c.c. decinormal HCl... | 10·0 | 3·3 | 2·2 | | | |
| 1·5 „ „ ... | 0 | 8·0 | 4·8 | | | |
| 2·0 „ „ ... | 8·6 | 3·0 | 1·5 | | | |
| 2·5 „ „ ... | 19·0 | 20·0 | 3·3 | 28·6 | 26·2 | |
| 3·0 „ „ ... | 33·3 | 39·3 | 1·7 | 33·3 | 42·4 | 4·6 |
| 4·0 „ „ ... | 75·0 | 39·4 | 1·8 | 75·0 | 39·4 | 5·5 |
| Average, exclusive of Control | 22·9 | 12·8 | 3·2 | 41·4 | 28·9 | 9·9 |

Table II.—Actual Number of Deaths in Fresh Eggs. Eggs of 2 days old and eggs of 10 days old with larger quantities of acid and alkali than were employed in the experiments, the results of which are shown in Table I. 40 eggs in each tumbler.

| | Fresh eggs. | | | | 2 days old. | | | | 10 days old. | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1st day. | 2nd day. | 3rd day. | 4th day. | 1st day. | 2nd day. | 3rd day. | 4th day. | 1st day. | 2nd day. | 3rd day. | 4th day. |
| Control ............... | 0 | 1 | 2 | 3 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 |
| 3 c.c. N/10 NaOH... | 0 | 2 | 11 | 25 | | | | | | | | |
| 4 ,, ,, ... | 2 | 5 | 17 | 29 | | | | | | | | |
| 5 ,, ,, ... | 0 | 5 | 34 | 36 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 2 |
| 6 ,, ,, ... | ? | 29 | 36 | all | | | | | | | | |
| 8 ,, ,, ... | ? | 10 | all | — | 0 | ? | ? | 28 | | | | |
| 10 ,, ,, ... | — | — | — | — | — | — | — | — | 0 | 1 | 2 | less than 10 |
| 15 ,, ,, ... | — | — | — | — | — | — | — | — | 0 | 2 | 3 | less than 10 |
| 20 ,, ,, ... | — | — | — | — | — | — | — | — | 0 | 2 | ? | 26 |
| 30 ,, ,, ... | — | — | — | — | — | — | — | — | ? | ? | ? | 26 |
| 3 c.c. N/10 HCl...... | 0 | 1 | 1 | 2 | | | | | | | | |
| 4 ,, ,, ...... | 0 | 0 | 1 | 3 | | | | | | | | |
| 5 ,, ,, ...... | 5 | 8 | 18 | 30 | 3 | 9 | 20 | 23 | | | | |

Strengths of 6, 8, 10, 15, 20 and 30 c.c. acid were also tried, all being fatal in less than 24 hours.

The figures for the 2nd, 3rd and 4th days are cumulative.

Table III.—Actual Number of Deaths among Fresh Eggs.

| | No. of eggs. | 2nd day. | 3rd day. | 4th day. | 5th day. | 6th day. | 7th day. | 8th day. | 9th day. | 10th day. |
|---|---|---|---|---|---|---|---|---|---|---|
| Control ......... | 20 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 3 | 5 |
| 1·0 c.c. N/10 NaOH | 23 | 0 | 0 | 0 | 1 | 1 | | | | |
| 1·5 ,, ,, | 21 | 0 | 1 | 1 | 2 | 2 | | | | |
| 2·0 ,, ,, | 20 | 1 | 4 | 4 | 5 | 5 | | | | |
| 2·5 ,, ,, | 22 | 4 | 4 | 4 | 4 | 4 | 6 | 6 | 6 | 7 |
| 3·0 ,, ,, | 22 | 3 | 5 | 5 | 5 | 6 | 6 | 6 | 7 | 7 |
| 4·0 ,, ,, | 25 | 3 | 8 | 9 | 11 | 11 | 11 | 12 | 12 | 12 |
| 1·0 c.c. N/10 HCl ... | 20 | 1 | 2 | 2 | 2 | 2 | | | | |
| 1·5 ,, ,, | 21 | 0 | 0 | 0 | 0 | 0 | | | | |
| 2·0 ,, ,, | 23 | 2 | 2 | 2 | 2 | 2 | | | | |
| 2·5 ,, ,, | 21 | 2 | 3 | 3 | 3 | 4 | 5 | 5 | 5 | 6 |
| 3·0 ,, ,, | 24 | 0 | 4 | 6 | 7 | 8 | 8 | 8 | 8 | 8 |
| 4·0 ,, ,, | 20 | 3 | 9 | 15 | 15 | 15 | 15 | 15 | 15 | 15 |

The figures in each column in this and the following two tables give the *total* number dead by the day in question.

Table IV.—Actual Number of Deaths among Eggs 2 Days Old.

| | No. of eggs. | 2nd day. | 3rd day. | 4th day. | 5th day. | 6th day. | 7th day. | 8th day. | 9th day. | 10th day. | 11th day. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Control .................... | 36 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1·0 c.c. N/10 NaOH | 28 | 0 | 0 | 0 | 0 | 0 | 0 | | | | |
| 1·5 ,, ,, | 23 | 0 | 0 | 0 | 0 | 0 | 0 | | | | |
| 2·0 ,, ,, | 32 | 0 | 0 | 0 | 0 | 0 | 0 | | | | |
| 2·5 ,, ,, | 38 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 3·0 ,, ,, | 55 | 0 | 0 | 0 | 0 | 4 | 7 | 7 | 7 | 7 | 8 |
| 4·0 ,, ,, | 56 | 1 | 2 | 2 | 5 | 10 | 14 | 23 | 23 | 23 | 27 |
| 1·0 c.c. N/10 HCl ... | 60 | 1 | 2 | 2 | 2 | 2 | 2 | | | | |
| 1·5 ,, ,, ... | 25 | 2 | 2 | 2 | 2 | 2 | 2 | | | | |
| 2·0 ,, ,, ... | 33 | 1 | 1 | 1 | 2 | 2 | 2 | | | | |
| 2·5 ,, ,, ... | 80 | 8 | 9 | 9 | 10 | 15 | 16 | 17 | 17 | 19 | 21 |
| 3·0 ,, ,, ... | 33 | 10 | 10 | 10 | 10 | 13 | 13 | 14 | 14 | 14 | 14 |
| 4·0 ,, ,, ... | 95 | 23 | 23 | 23 | 26 | 31 | 34 | 34 | 34 | 34 | 34 |

Table V.—Actual Number of Deaths among Eggs 10 Days Old.

| | No. of eggs. | 2nd day. | 3rd day. | 4th day. | 5th day. | 6th day. | 7th day. | 8th day. | 9th day. | 10th day. | 11th day. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Control ......... | 245 | 0 | 0 | 1 | 4 | 4 | 5 | 6 | 6 | | |
| 1·0 c.c. N/10 NaOH | 165 | 0 | 2 | 4 | 6 | 6 | 7 | | | | |
| 1·5 „ „ | 105 | 0 | 1 | 1 | 1 | 1 | 3 | | | | |
| 2·0 „ „ | 110 | 1 | 1 | 1 | 1 | 1 | 1 | | | | |
| 2·5 „ „ | 100 | 0 | 0 | 1 | 1 | 1 | 3 | | | | |
| 3·0 „ „ | 80 | 0 | 0 | 0 | 1 | 1 | 2 | 2 | 2 | 2 | 2 |
| 4·0 „ „ | 160 | 1 | 1 | 2 | 5 | 7 | 15 | 30 | 39 | 43 | |
| 1·0 c.c. N/10 HCl ... | 135 | 0 | 1 | 1 | 2 | 2 | 3 | | | | |
| 1·5 „ „ ... | 105 | 0 | 0 | 2 | 2 | 4 | 5 | | | | |
| 2·0 „ „ ... | 155 | 0 | 0 | 0 | 0 | 0 | 1 | | | | |
| 2·5 „ „ ... | 120 | 0 | 1 | 2 | 3 | 3 | 4 | | | | |
| 3·0 „ „ ... | 175 | 0 | 3 | 3 | 3 | 3 | 3 | 3 | 5 | 6 | 8 |
| 4·0 „ „ ... | 110 | 0 | 1 | 1 | 2 | 2 | 2 | 3 | 5 | 5 | 6 |

Table VI.—Percentages of 10-Days-old Eggs which succeeded in Hatching.

| | |
|---|---|
| Control ....................... | 98·4 |
| +1·0 c.c. N/10 NaOH ...... | 96·4 |
| 1·5 „ ...... | 99·0 |
| 2·0 „ ...... | 99·1 |
| 2·5 „ ...... | 99·0 |
| 3·0 „ ..... | 98·7 |
| 4·0 „ ...... | 95·6 |
| +1·0 c.c. N/10 HCl ......... | 98·5 |
| 1·5 „ ......... | 96·2 |
| 2·0 „ ......... | 100·0 |
| 2·5 „ ......... | 97·5 |
| 3·0 „ ......... | 98·3 |
| 4·0 „ ......... | 98·2 |
| Dimethyl ..................... | 39·7 |
| Phenol-phthalein ............ | 98·8 |

Table VII.—Showing the Effect of Indicators upon the Eggs of Pleuronectes.

| Batch. | Indicator. | No. of eggs. | Number dead at end of— | | | | | | | | | | Percentage of dead at end of— | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 2 days. | 3 days. | 4 days. | 5 days. | 6 days. | 7 days. | 8 days. | 9 days. | 10 days. | 11 days. | 2 days. | 3 days. | 4 days. | 5 days. | 6 days. | 7 days. | 8 days. | 9 days. | 10 days. | 11 days. |
| Fresh eggs | Dimethyl ......... | 20 | 3 | 3 | 5 | 7 | 8 | 9 | 12 | 12 | 18 | — | 15 | 15 | 25 | 35 | 40 | 45 | 60 | 60 | 90 | |
| | Phenol-phthalein | 28 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | — | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3·6 | 3·6 | |
| | Control ............ | 20 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 3 | 5 | — | 5 | 5 | 5 | 5 | 5 | 5 | 10 | 15 | 25 | |
| 2 days old | Dimethyl ......... | 70 | 0 | 0 | 0 | 2 | 4 | 8 | 15 | 20 | 64 | 70 | 0 | 0 | 0 | 2·8 | 5·7 | 11·4 | 21·4 | 28·6 | 91·4 | 100 |
| | Phenol-phthalein | 50 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 3 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 4 | 4 | 6 |
| | Control ............ | 36 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 10 days old | *Dimethyl ......... | 63 | 2 | 2 | 2 | 6 | 25 | 38 | 46 | 60 | 63 | — | 3·2 | 3·2 | 3·2 | 9·5 | 39·7 | 60·3 | 73 | 95·2 | 100 | |
| | †Phenol-phthalein | 83 | 0 | 0 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | — | 0 | 0 | 1·2 | 1·2 | 1·2 | 1·2 | 2·4 | 2·4 | 2·4 | |
| | Control ............ | 245 | 0 | 1 | 2 | 5 | 5 | 6 | 7 | 7 | — | — | 0 | 0·4 | 0·8 | 2·0 | 2·0 | 2·4 | 2·9 | 2·9 | | |

* 25 of these eggs reached the larval stage.
† 82 „ „

### Table VIII (Brood 1).

Showing the Effect of Indicators upon Echinus Eggs in Solutions Containing Varying Quantities of NaOH in 200 c.c Sea-water.

| No. | Indicator. | Amount of N/10 NaOH. | After 4 hours. | 17 h. 45 m.* | 24 h. 50 m. | 29 h. 15 m. | 41 h. 45 m. | 70 h. 30 m. | 91 h. 15 m. | 114 h. 0 m. |
|---|---|---|---|---|---|---|---|---|---|---|
| | | c.c. | | | | | | | | |
| 1 | None ...... | 0 | 4 cells.............. | $1\frac{1}{2}$ Blastulæ ... | B., a few moving | All active | B., rapid movement | B., commencing G., mostly dead | Dead and degenerating | |
| 2 | „ ...... | 0·2 | „ .............. | $1\frac{3}{4}$ „ ... | „ „ | | „ „ | Early G., mostly dead | „ „ | |
| 3 | „ ...... | 0·4 | „ .............. | $1\frac{3}{4}$ „ ... | „ „ | | | Commencing G., all dead, granular | „ „ | |
| 4 | „ ...... | 0·8 | 4, 6, and 8 irreg. | $1\frac{7}{8}$ „ ... | „ „ | „ „ | „ „ | G., active ........ | Active, late G.‡ | |
| 5 | „ ...... | 1·4 | 4 irreg. ............ | $1\frac{3}{4}$ „ ... | B., motionless | | | „ ........ | ½ dead, not so advanced as preceding | Very active |
| 6 | „ ...... | 2·0 | 2, and a few 4 ... | $1\frac{7}{8}$ „ ... | „ „ | „ „ | B. and some G. | „ † ...... | Dead, but better formed and more advanced than preceding | |
| 7 | Dimethyl | 0 | $\frac{4}{5}$ 4-celled, $\frac{1}{5}$ 8-celled | $1\frac{7}{8}$ „ ... | B., some moving | „ „ | B., swimming. | Early G., dead, granular | Dead and degenerating | |
| 8 | „ | 0·2 | „ „ | $1\frac{3}{4}$ „ ... | „ „ | | | „ „ | „ „ | |
| 9 | „ | 0·4 | 4, and occasional 2 and 8 | $1\frac{3}{4}$ „ ... | „ „ | | | Early G., not very active | Dead | |
| 10 | „ | 0·8 | 4, and $\frac{1}{10}$ above 4 | $1\frac{7}{8}$ „ ... | Several moving | Some active some encapsuled (?) | „ „ | Early G., fairly active | More advanced than preceding, but dead | |
| 11 | „ | 1·4 | 4 irreg., some 8 and 12 | $1\frac{3}{4}$ „ ... | A few moving | | | Early G., slightly active | „ „ | Degenerating |
| 12 | „ | 2·0 | 4, 6, 8, and more | $1\frac{3}{4}$ „ ... | „ „ | „ „ | Gastrulæ forming | „ „ | Well advanced, dead | Not yet degenerating |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 13 | Phenol-phthalein | 0 | 4, and $\frac{1}{3}$ 8 ......... | $1\frac{3}{4}$ Morulæ...... | M. and $\frac{1}{30}$ B. | Same as before | Degenerated | Dead and degenerated | | |
| 14 | ,, | 0·2 | Irreg., a few 2, mostly 4 | 12 cells less divided than preceding | M. | | ,, | ,, ,, | | |
| 15 | ,, | 0·4 | 4 and over, several 2, irreg., and breaking up | Irreg. division, $1\frac{3}{8}$ M. | M., irreg. | | | ,, ,, | | |
| 16 | ,, | 0·8 | Many, 3 irreg., a few 2 | M. irreg., $1\frac{3}{4}$... | ,, ,, | ,, ,, | | Dead | | |
| 17 | ,, | 1·4 | 4, $\frac{1}{3}$ more, very irreg. | M., $1\frac{3}{4}$ more cells than preceding | ,, ,, | | | ,, | | |
| 18 | ,, | 2·0 | Very irreg., 2, 3, and 4 | ,, ,, | Several badly-formed B. | ,, ,, | B., motionless | ,, | | |

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* Measurements arbitrary by linear marks on paper.

† Small amount of formalin added by mistake after examination.

‡ Bottle unfortunately broken after examination.

Table IX.—Effect of Indicators on Echinus Eggs in Solutions containing Varying Quantities of KOH in 200 c.c. Sea-water.

(Brood 2.)

| No. | Indicator. | Amount of N/10 KOH. | After 4 hrs. 30 mins. | 12 hrs. 30 mins. | 24 hrs. 30 mins. |
|---|---|---|---|---|---|
| | | c.c. | | | |
| 1 | None | 0 | 2's | Morulæ and early blastulæ, 17* | Dead |
| 2 | „ | 1·0 | 2, occasional 3 and 4 | Early blastulæ, more advanced | Blastulæ, some early gastrulæ |
| 3 | „ | 1·5 | „ „ | Blastulæ, 26 | Dead |
| 4 | „ | 2·0 | „ „ | Blastulæ, few developed | Commencing gastrulæ, dead |
| 5 | „ | 3·0 | Nearly all 1's, irregular, granular | No morulæ, irregular 2's and up to 16's | Poorly developed, some morulæ, dead |
| 6 | „ | 4·0 | Irregular division, very few cells visible | Broken up | |
| 7 | „ | 5·0 | None divided | „ | |
| 8 | Dimethyl | 0 | 2, some incomplete 4 | Morulæ and early blastulæ, 18 | Blastulæ, dead |
| 9 | „ | 1·0 | 2's | Blastulæ, 28 | Blastulæ and occasional early gastrulæ, dead |
| 10 | „ | 1·5 | 2, a few incomplete 3 | „ | „ „ „ |
| 11 | „ | 2·0 | 2, irregular division into 3 | Slightly larger than preceding | „ „ „ |
| 12 | „ | 3·0 | Same as 5 | Irregular, no blastulæ yet formed | Morulæ or earlier stage, dead |
| 13 | „ | 4·0 | Irregular and breaking up | 16 cells and less, irregular | Unsegmented or broken up |
| 14 | „ | 5·0 | 1's, a few irregular divisions | Irregular and broken up | |
| 15 | Phenol-phthalein | 0 | 2, a few 4 | Morulæ and less, 16 irregular | Morulæ, dead |
| 16 | „ | 1·0 | 2, 4, and incomplete further divisions | Early blastulæ, 20 | „ „ |
| 17 | „ | 1·5 | Many 1, some 2, and some incomplete 4 | „ „ 12 | Morulæ or earlier stages, dead |
| 18 | „ | 2·0 | 2 and irregular 3 and 4 | „ „ 16 | Better than last |
| 19 | „ | 3·0 | Same as 5 | Few morulæ, mostly 2, 3, 8, and 16 | Same as last but one |
| 20 | „ | 4·0 | Same as 13 | Very irregular, but nearly all 1 | Same as before |
| 21 | „ | 5·0 | Same as 7 | Single cells, breaking up | |

* Numbers in this column give number of cells counted in the circumference of the blastula.

Table X.—Effect on Development of Echinus Eggs of 0·5 Per Cent. Alcoholic Solution of Phenol-phthalein in Varying Strengths, added in each Case to 200 c.c. Sea-water.

(Brood 3.)

| No. | Amount of indicator. | 3 hrs. 30 mins. | 19 hrs. | 47 hrs. 30 mins. | 71 hrs. | 114 hrs. |
|---|---|---|---|---|---|---|
| 26 | c.c.<br>0·05 | Some complete 2, some incomplete 3 and 4 | $\frac{1}{5}$ blastulæ, $\frac{1}{5}$ morulæ, rest earlier | Morulæ and a few developing blastulæ | Not well developed …… | Degenerating blastulæ |
| 27 | 0·1 | Mostly 2, also 3 and 4… | $\frac{1}{3}$ at 2, rest morulæ and blastulæ about equal | Slightly earlier than last | … … | $\frac{3}{4}$ countable, 2 morulæ, a few blastulæ |
| 28 | 0·15 | 2, several 4, some irregular | No blastulæ, $\frac{1}{2}$ badly-formed morulæ, rest 2, 3, 4, etc. | Early morulæ, a few still remain unsegmented | Only slightly developed | Less advanced and more irregular than last |
| 29 | 0·2 | … … | Less advanced ………… | A few morulæ, about $\frac{1}{2}$ still unsegmented | … … | Singles to early morulæ, nearly all countable |
| 30 | 0·3 | … … | $\frac{9}{10}$ not beyond 2's, rest irregular morulæ | Still more singles than last | Mostly 1, 2 and 3……… | Singles, 2, 4, 6, $\frac{1}{10}$ early morulæ, less advanced than last |
| 31 | 0·4 | 1's, a few incomplete 2 | $\frac{9}{10}$ single, scarcely any 2, rest irregular 4, 8 and 12 | $\frac{3}{4}$ singles, rest early divisions, irregular | … … | Less advanced still, $\frac{1}{20}$ early morulæ, nearly all single but some irregular attempts at division |

# *The Mammalian Cerebral Cortex, with Special Reference to its Comparative Histology. I. Order Insectivora.*—Preliminary Communication.

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(Communicated by Dr. F. W. Mott, F.R.S. Received July 28,—Read December 14, 1905.)

The results and conclusions brought forward in this paper form a portion of the outcome of an extensive investigation dealing with the cortex cerebri in various orders of mammals. The work has special reference to the neopallium only, and has for its prime purpose an endeavour to shed some further light upon the functional significance of the cerebral cortical lamination.

## *Animals Examined and Methods of Study.*

The brains of the animals belonging to this order examined are :—

1. The Mole (*Talpa Europea*). 2. The Shrew (*Sorex vulgaris*). 3. The Hedgehog (*Erinaceus Europeus*).

The cerebral cortex has been examined by means of complete series of sections cut in almost every possible direction and stained by one or other modification of the Nissl method.

As part of the method of study throughout the entire investigation, the natural habits of the animals examined, and their educability, as far as facts relating to the latter are available, have been considered when attempting to correlate structure and function.

## *Macroscopic Appearances and Microscopic Furrows.*

All are almost smooth highly macrosmatic brains. That of the Hedgehog is one of the simplest mammalian brains. It presents in addition to the rhinal fissure a short presylvian furrow; the latter is not found macroscopically in the Mole and Shrew, but is seen on microscopic examination of sections. By this method also, in the Mole only, two shallow more or less longitudinal curved furrows can be traced on the dorso-lateral aspect of the hemisphere, which appear to represent foreshadowings of the corono-lateral and supra-sylvian sulci.

Signs of greater differentiation of the neopallium of the Mole, as compared with the Hedgehog especially, are further exhibited on microscopic examination of the structure of the cortex. The optic nerves in the Mole and

Shrew are reduced to small threads. In the Hedgehog these nerves are considerably larger. In all three the fifth nerves are relatively very large.

### *The Lamination of the Neopallium.*

General Remarks.—The classification of the cortical layers adopted by the writer is that introduced by J. Shaw Bolton. The latter considers that the human cerebral cortex is constructed upon a five-layered type—viz., I, Molecular; II, Pyramidal; III, Granular; IV, Inner line of Baillarger; V, Polymorphic. Of these only three are primarily cell layers—viz., the pyramidal, granular, and polymorphic, Layers I and IV being primarily fibre layers, although containing nerve cells—the cells of Cajal in Layer I, and the Betz cells (psycho-motor region) or solitary cells of Meynert (other regions) in Layer IV. The outstanding features of this classification are: (1) The recognition of the granular layer as separating the true pyramidal *layer* above from the more or less pyramidal *shaped* cells which may be found below this layer, for the cells of Layer IV are not "pyramidal" cells at all, the Betz cells in the psycho-motor area constituting "the origin of the important tract for skilled voluntary movement," whilst the solitary cells of Meynert in other regions "probably possess a somewhat analogous function." (2) The consideration of the pyramidal layer as forming *one* layer developmentally and functionally.

Bolton, as the result of his studies of the development of the human cerebral cortical layers, and of their depth in the normal individual as well as in various degrees of amentia and dementia, has come to the following conclusions as to the functions of the three primary cell layers. The pyramidal layer "subserves the psychic or associational functions of the cerebrum." The granule layer "probably subserves the reception or immediate transformation of afferent impressions, whether from the sense organs or from other parts of the cerebrum," whilst the fifth, or polymorphic layer, "probably subserves the lower voluntary functions of the animal economy."

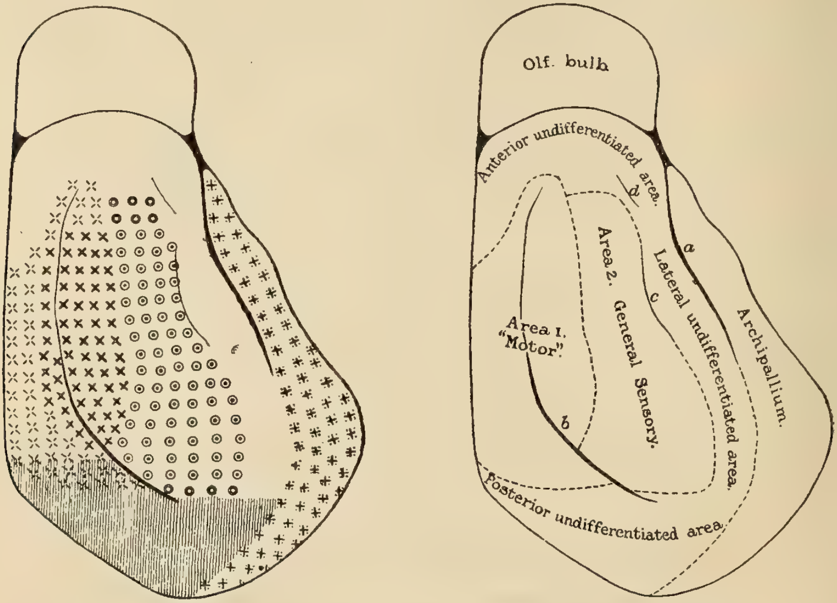
When dealing with the mammalian cortex generally one or two further explanations are necessary.

The term "granular" is used in a wide generic sense and as indicative of a certain cortical layer rather than of the cell constituents of this layer, which latter, in an adult animal, may take the form of angular, quadrilateral, stellate or even small pyramidal-shaped cells, or a mixture of these elements. In some regions of the cortex in certain animals these elements of the granular layer may be scattered and comparatively few in number; yet their recognition is of importance, for such provides the means by which the lower limits of the true pyramidal layer may be determined. Owing to the difficulty experienced in accurately separating the fourth and fifth layers (which tend to intermingle) in the cortex of some animals, the writer prefers to speak of these layers together as *infra-granular.* It is also proposed for the sake of definiteness to term the true pyramidal layer (*i.e.*, Layer II) the *supra-granular* layer.

### *Areas in the Neopallium.*

The appearances of the cortex in the Mole and Shrew being very similar, the following, concerning the cortex of the Mole, may be taken as applying also to the Shrew excepting when the latter is specially mentioned:—

1. *Dorso-lateral Surface.*—In the Mole this region presents two main and distinct types of cortical structure, with certain areas of comparatively undifferentiated cortex (fig. 1).



Olf. bulb
Anterior undifferentiated area.
d
Area 2. General Sensory.
Lateral undifferentiated area.
a
c
Archipallium.
Area 1. "Motor."
b
Posterior undifferentiated area.

FIG. 1.—Dorso-lateral view of the Right Hemisphere of the Mole.

*Left-hand figure.* × Area 1, "Motor"; ⋇ the same, but less characteristic; ⊙ Area 2, General Sensory; ◎ the same, but less characteristic; ⁜ Archipallium.

The anterior, lateral, and posterior areas of undifferentiated cortex are left blank excepting the portion of neopallium represented as shaded, which is thinner than the remainder.

*Right-hand figure.* *a*, rhinal fissure; *b*, *c*, and *d*, probable representatives respectively of the corono-lateral, supra sylvian, and presylvian sulci; *b*, *c*, and *d*, vary much in individual distinctness in different hemispheres. The figure is a composite one.

*Area I: Motor.*—This extends antero-posteriorly from a short distance behind the anterior pole to about the posterior quarter of the hemisphere, and laterally from the dorso-mesial margin (or more or less close to this anteriorly and overlapping this posteriorly), to about half-way between the dorso-mesial edge and the rhinal fissure, the lateral limits varying somewhat at different points (figs. 1 and 2).

*Area II: General Sensory.*—This occupies an extensive region lateral to Area I, but does not reach as far as the rhinal fissure, being separated from the latter by a zone of undifferentiated cortex (fig. 1).

The features of Area I are less characteristic mesially, anteriorly and posteriorly, and of Area II anteriorly and posteriorly. These two fields appear in every way to be the best developed areas of the neopallium and to be amongst the oldest phylogenetically. Owing to their different histological appearances, Areas I and II in the Mole can be readily delimited. From the relatively greater numbers and prominence of the large cells (homologues of Betz cells) in Layer IV, it is concluded that Area I possesses especially motor attributes, and that on account of the greater development of the granular layer throughout Area II, the latter is sensory in function. It is also thought that probably Area II is largely concerned with kinæsthetic impressions, these appearing to constitute a considerable part of the basis of such intelligence as these animals possess.

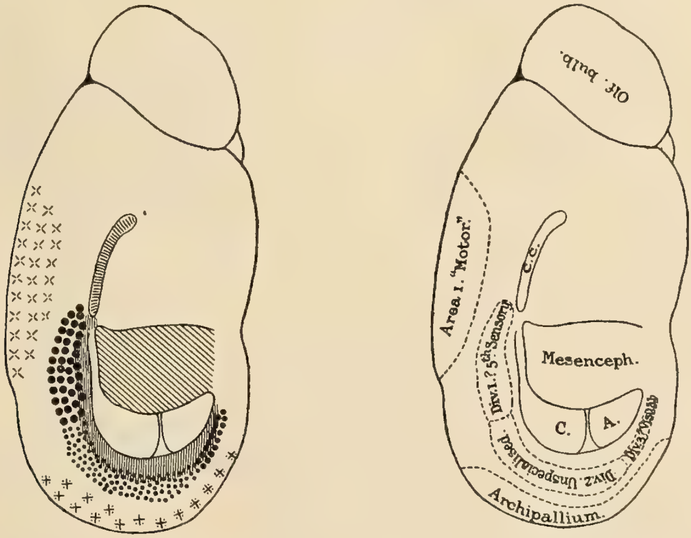


Fig. 2.—Postero-mesial aspect of the Left Hemisphere of the Mole, to illustrate especially the position and relations of the postero-mesial region of cortex characterised by a well-marked granular layer. The right-hand figure is explanatory of that on the left.

In the Hedgehog, although interpretation of the cortical structure is much more difficult than in the Mole, a similar large field of comparatively well-developed cortex can be distinguished on the dorso-lateral and mesial aspects, occupying the same relative position and being of about the same relative size, as are Areas I and II taken together in the Mole. No satisfactory criteria, however, have been found from cell lamination alone by the aid of which this region can be divided into Areas I and II as in the Mole. Hence, it is considered that although there may be some attempt at differentiation of these areas in the Hedgehog, it is probably more correct to regard the entire field in the latter as a combined sensori-motor area.

The explanation which suggests itself of these structural differences is that they stand in relation to the fact that the Mole and Shrew are possessed of more numerous and better motor accomplishments than the Hedgehog, and so the former have a more specialised zone of "motor" cortex, the latter being a comparatively lethargic animal, and dependent for its survival rather upon its protective armour of spines than upon its activity.

*Areas of Undifferentiated Cortex.*—In the anterior (extending also to the mesial), lateral, and posterior portions of the neopallium are three fields of moderate size in all three animals, to which, from the want of any special feature in their lamination, no attempt is made at the present time to attach any peculiar functional value. They are regarded as indifferent or unspecialised regions of cortex.

The area of the cortical distribution of the eighth nerve has not been definitely localised. By analogy one would expect to find this probably in about the middle and towards the anterior part of the outer portions of the field termed Area II in the Mole, or just external to this, and in the corresponding region in the Hedgehog.

2. *Anterior Surface.*—Occupying almost the whole of the anterior and inferior aspect of the frontal pole and curving forwards to join the olfactory bulb, is a small area of comparatively well-developed cortex, sharply marked off from the less differentiated cortex posteriorly. This is of about the same relative size in the Mole and Hedgehog, and probably constitutes a neopallial representation of the olfactory sense.

3. *Mesial Surface.*—This may be divided into (1) the straight anterior portion, and (2) the curved postero-ventral portion which arches over and round the mesencephalon and hippocampus (figs. 2 and 3).

(1) The straight portion presents anteriorly an area of undifferentiated cortex continuous with that on the dorsal aspect, posteriorly a field with modified motor characters joining that in the dorsal surface, and between these an area with some indefinite sensory characters. Similar areas are seen in the Hedgehog.

(2) The more definite lamination (figs. 2 and 3) occupies the remainder of the posterior part of the straight mesial surface and a large part of the curved postero-mesial and ventral surfaces. This region, from sections taken in various directions, was found to be distinguished by an exceedingly definite granular layer, and it appears to be a portion of neopallium inserted as a curved tongue between the mesial portion of Area I (in Mole) and the cortex behind and below this, including archipallium on the one hand, and the posterior part of the corpus callosum and hippocampus on the other. The area is not of the same shape in the Mole as in the Hedgehog, owing to the hemisphere being moulded in a different way in the two animals. This region, although characterised throughout by the well-marked granular layer referred to, presents certain differences of detail, owing to which it has been separated into three divisions in each animal. The anterior and superior portion (Division 1) is of about the same relative size in the Mole as in the Hedgehog. In both there are good granular and infra-granular layers, and in the Hedgehog a shallow but definite supra-granular layer. The middle portion (Division 2) is relatively

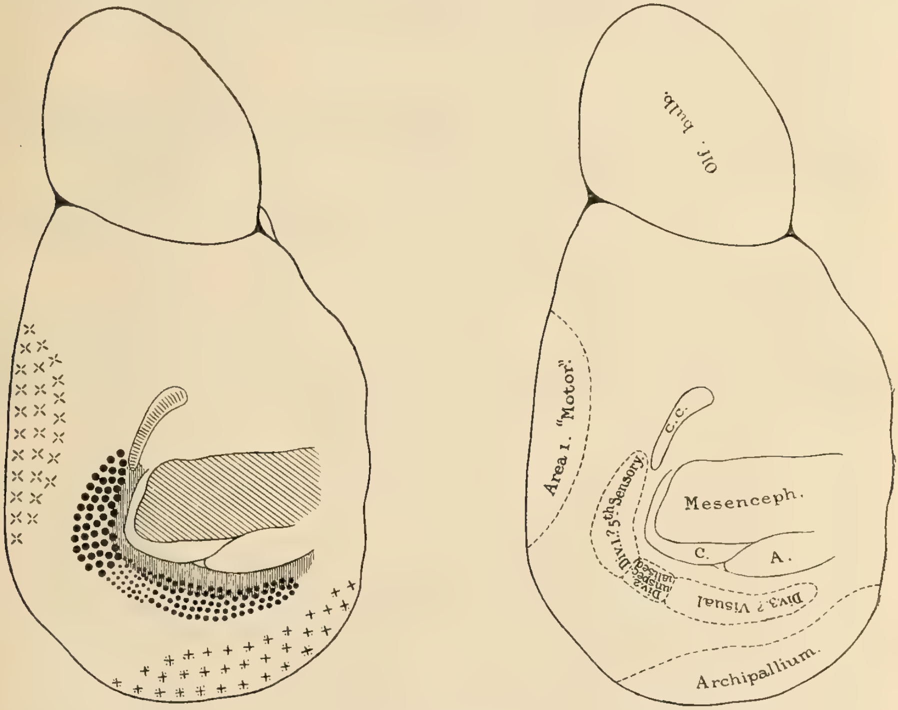


Fig. 3.—Postero-mesial aspect of the Left Hemisphere of the Hedgehog, for comparison with fig. 2 (Mole).

larger in the Mole than in the Hedgehog, but in both, comparatively to Divisions 1 and 3, it presents unspecialised features. The inferior portion (Division 3) is relatively not only considerably larger in the Hedgehog than in the Mole, but in the former it is a more histologically distinct field, showing not only a well-formed "granular" layer (the cellular elements in which, however, are mostly angular or small pyramidal in shape), but a comparatively good infra-granular and a definite, though shallow, supra-granular layer. In the Shrew this division is rather better developed than in the Mole.

Owing to the presence throughout this region of such a deep and definite granular layer, it is concluded that this field is sensory in function, and regarding it the following suggestions are made :—

(*a*) The area is much too large to be concerned only with the cortical distribution of the optic nerves, which are relatively so minute in these animals, especially in the Mole.

(*b*) The inferior portion (Division 3) only is visual. In the Mole this portion is a mere vestige; in the Hedgehog it is better developed and relatively larger, having apparently extended upwards somewhat and encroached upon the area of unspecialised granular cortex (Division 2) as compared with the Mole.

(*c*) The middle and superior portions (Divisions 2 and 1) may correspond to the large infra-calcarine area of certain relatively higher mammals (*e.g.*, Ungulata and Carnivora), in which, owing to the greater development of the visual faculty, the inferior portion (Division 3, visual) has, so to speak, extended upwards, backwards, outwards, and forwards so as to overlie the middle and superior portions, and has become the calcarine region.

(*d*) The relatively well-developed superior and anterior portion (Division 1) of the two specialised divisions in both Mole and Hedgehog may be concerned with the cortical distribution of the fifth sensory nerve. On account of the importance of the fifth sensory nerve as an avenue of information to these mammals through snout or vibrissæ touch, or both, and, in view of the large size in them of the fifth nerve, it seems probable that the sensory portion of this nerve should have a very special cortical representation.

### *The Cerebral Cortical Layers.* (Neopallium.)

Although the total depth of the cortex in the best developed regions is different in the Mole, Shrew, and Hedgehog, the relative depth of the separate layers, supra- and infra-granular particularly, appears to be about the same in all. The following micrometric measurements of the cortical layers in three areas of the Mole's cortex, which have been kindly furnished by Dr. J. S. Bolton, may be taken as fairly typical also of the relative differences in depth of the supra- and infra-granular layers especially, in the corresponding regions in the other two animals.

Micrometric Measurements of the Cortex of the Mole.*

| | Area 1.—Motor (average of 23). | | Area 2.—General sensory (average of 17). | | Lateral area (average of 7). | |
|---|---|---|---|---|---|---|
| | mm. | mm. | mm. | mm. | mm. | mm. |
| Layer I.—Molecular | 0·162 | | 0·144 | | 0·095 | |
| „ II.—Supra-granular | 0·092 | 0·320 | 0·093 | 0·367 | 0·095 | 0·320 |
| „ III.—Granular | 0·228 | | 0·274 | | 0·225 | |
| „ IV.—Infra-granular | 0·147 | 0·304 | 0·176 | 0·377 | Not separated | 0·471 |
| „ V. | 0·157 | | 0·201 | | | |
| Total | 0·786 mm. | | 0·888 mm. | | 0·886 mm. | |

It will be observed that in Area I the supra-granular layer (II) is less than one-third the depth of the infra-granular layer (IV and V), and that in Area II it is only about one-quarter of the depth of the latter. If Layers II and III (supra-granular and granular) are taken together they only approximately equal in depth the infra-granular.

A very abbreviated comparative summary of these measurements in the case of the Mole, and those of Bolton† from the prefrontal cortex in the sixth month's human fœtus, the full-time new-born child and the normal human adult, reveals the following facts, shown approximately in fig. 4:—

(1) The granular layer is of approximately the same depth in the Mole (Areas I and II) as in the normal human adult prefontal cortex.

(2) The supra-granular (pyramidal) layer: (*a*) in the sixth month's human fœtus is nearly two and a-half times the depth of that of the Mole (Areas I and II); (*b*) in the full-time child it is five to six times the depth of that of the Mole; (*c*) in the normal human adult nine times the depth.

(3) The infra-granular layer.—In the sixth month's fœtus and full-time

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[*] With regard to the lateral area of undifferentiated cortex, the cellular elements in which are comparatively unspecialised, it is obvious that its increase in depth as compared with Area I is due chiefly to the infra-granular layers (IV and V), the portion of the cortex from which the measurements were taken being in the neighbourhood of the rhinal fissure, and so forming what would correspond to the apex of a convolution.

[†] Bolton's observations as to the ontogenetic development of the cerebral cortical layers have been confirmed by the writer in the cases of a fourth, sixth, and eighth month human fœtus, and the same relative order of development of the layers has been found by him to hold good with regard to several fœtal and new-born lower mammals belonging to different orders.

child the combined fourth and fifth layers are little over the depth of those layers in the Mole. In the normal human adult these combined layers are only a little over half as deep again as in the Mole (1·56 to 1).

The increase in depth of the human cortex cerebri as compared with that of the Mole is therefore *very* largely due to increase in the supra-granular layer.

### *Conclusions as to the Functional Significance of the Supra-Granular and Infra-Granular Cortical Layers.*

Bolton's views as to the functional value of these layers have already been briefly stated (p. 151). The following conclusions, which apply only to mammals, form a complement of them from the point of view of the Insectivora and of the lower mammals belonging to various other natural orders so far examined.

1. The infra-granular layer (IV and V) (omitting the constituent cells which possess motor or analogous functions), which is relatively so fully developed at birth, is concerned especially with the associations necessary for the performance of the instinctive activities, that is, all those which are innate and require no experience or education. These involve many complex actions, such as the seeking appropriate shelter and protection (*e.g.*, tunnelling of Mole), the hunting for food—each after his own kind—and the quest of the opposite sex. Such more or less stereotyped activities may show signs of improvement, firstly as the result of the perfection by use of an inherited mechanism, and secondly as the result of the intermingling of activities for which it is concluded that the supra-granular layer is responsible. In the latter case, however, the activities would merge into those which are more properly described as habitual intelligent or into the class of incomplete instincts (Lloyd Morgan), or mixed instincts (Romanes).

2. The supra-granular (pyramidal) layer subserves the higher associations, the capacity for which is shown by the educability of the animal. It has therefore to do with all those activities which it is obvious that the animal has acquired by individual experience, and with all the possible modifications of behaviour which may arise in relation to some novel situation, hence with what is usually described as indicating intelligent as apart from instinctive acts.

In practical animal behaviour the two sets of processes are probably more or less constantly interwoven, the higher activities (supra-granular layer) coming to the aid of the lower as far as the capability of the animal allows. In the case of the lower mammals (*e.g.*, Insectivora) the limits of this capability are comparatively soon reached, and, correspondingly, these

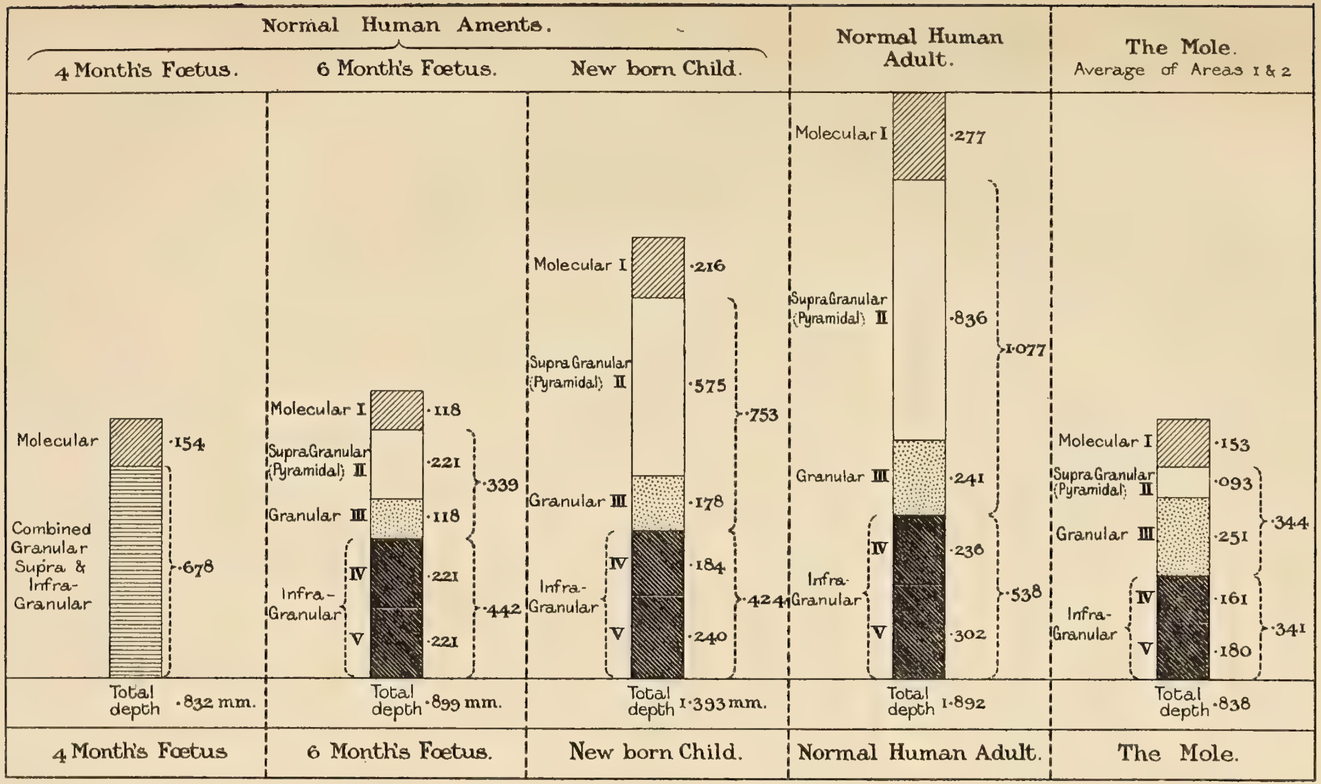

FIG. 4.—Illustrates approximately the relative depths of the Cerebral Cortical Layers in normal Human Aments (4 and 6 months' fœtus and new born child), the normal Human Adult and the Mole.

The micrometric measurements in the first four cases are taken from "The Histological Basis of Amentia and Dementia" ('Archiv of Neurology,' vol. 2, 1903) by J. Shaw Bolton; those of the cortex of the Mole are also by Dr. Bolton. Supragranular (pyramidal) layer left blank; infragranular (IV and V) shaded darkly.

mammals possess a relatively poor supra-granular layer. Many of these lower mammals have adopted a safe mode of life, others have resorted to fecundity. With these, which may for present purposes be termed extraneous aids to survival, their essentially instinctive activities have been relatively sufficient to ensure their continued existence. There has therefore in these mammals been little necessity for the development of a supra-granular layer, the infra-granular providing most of the necessary cortical physical basis required for practical behaviour.

The infra-granular layer, with the reservation to which reference has been made, thus constitutes the earlier developed and more fundamental associational system of the cerebral cortex; the supra-granular, a higher and accessory system, super-added, and of any considerable functional importance only in certain regions in lower mammals such as the Insectivora.

In view of the above conclusions, attention may be briefly directed to the following points. Areas I and II—motor and general sensory—in the Mole and Shrew (and the combined field in the Hedgehog) appear in every sense to be the most completely developed regions of neopallium which these animals possess, and are the only areas in the two former animals having a supra-granular layer of any considerable depth and complexity as regards its individual cell elements. In the Hedgehog, in the area which is believed to have visual functions, there is as regards individual cells a moderately well-developed, though thin, supra-granular layer, whilst this is practically absent in the comparatively blind Mole and Shrew in the analogous region.

*Note to* Fig. 4.—The infra-granular layer in the 6-months' fœtus and new-born child were practically of the same depth in the specimens measured, viz., ·442 to ·424. In the figure the darkly-shaded part (infra-granular layer) in the third column is therefore represented somewhat too deep.

# *On the Microsporangia of the Pteridosperms.*

By Robert Kidston, F.R.S. L. and E., F.G.S.

(Received November 15,—Read December 14, 1905.)

(Abstract.)

The first point considered is the question of the identity of *Lyginodendron Oldhamium* and *Sphenopteris Höninghausi*, Brongt. Although this seems to be generally accepted, it has not, as far as the author knows, ever been demonstrated and from continental botanists a clear proof of their identity may be justly demanded. The form, nervation, mode of attachment of the sterile pinnules, the presence of spines on the pinnules and stems and the sclerenchymatous net-like bands in the outer cortex are shown to be similar in both, and further, that as a petrifaction, *Lyginodendron Oldhamium* on the one hand, and as an impression, *Sphen.* (*Crossotheca*) *Höninghausi* on the other, each in their respective condition, are the only two plants occurring on the same horizon what each possess all these characters.

The structure of the *microsporangia* of *Sphen.* (*Crossotheca*) *Höninghausi* is then described in detail, of which a preliminary account has already been given.* This is followed by a description of the *microsporangia* of *Crossotheca Hughesiana*, n. sp., which possesses all the essential characters of the *microsporangia* of *C. Höninghausi*, differing only in its larger size and the limb of the fertile pinnules being cordate, not oval, as in *C. Höninghausi*.

The last part of the paper deals with the affinities of the *Cycadofilices*. Beginning with the Upper Devonian, where the first satisfactory evidence of the occurrence of "ferns" or "fern-like" plants is found, the fructification of Archæopteris is discussed and the conclusion arrived at that it agrees much more with *microsporangia* than with true fern *sporangia*. The "ferns" of the Culm are next considered, where the only evidence of "ferns" is found in the occurrence of a few sporangia with an annulus of more than one row of cells. The presence of the *Cycadofilices* on this *horizon* is very conspicuous; the *Botryopterideæ* also occur if one is justified in regarding *Zygopteris* as their petioles. The annulate sporangia from this horizon are regarded as being more probably related to the *Botryopterideæ* than to the true ferns.

The "ferns" or "fern-like" plants of the Upper Carboniferous are next reviewed. In the *Lanarkian series* or lowest division of the Upper Carboniferous, which includes the Halifax hard bed—it is pointed out, that though

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* 'Roy. Soc. Proc.,' vol. B. 76, p. 358, 1905.

*Cycadofilices*, *Pteridosperms* and *Botryopteris* occur, there is no satisfactory evidence of the existence of a true fern on this horizon. In the succeeding *Westphalian series,* true ferns possibly exist in such genera as *Hymenophyllites*, *Oligocarpia*, *Senftenbergia*, and *Kidstonia.* Also the *Marattiaceæ* are present in *Asterotheca* and some allies. In the *Radstockian series* and *Upper Coal Measures* of the continent, all these groups are probably present, but the *Marattiaceæ* assume here a very important place.

The conclusion is, therefore, come to that the *Cycadofilices*, which long antedated the advent of true ferns, cannot have been derived from them but are themselves the oldest type of fern-like plant at present known. In regard to the true ferns it seems probable that they may have been derived from the *Botryopterideæ.*

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# *Some Observations on* Welwitschia mirabilis, *Hooker, f.*

By H. H. W. Pearson, M.A., F.L.S., "Harry Bolus" Professor of Botany in the South African College, Cape Town.

(Communicated by A. C. Seward, F.R.S. Received September 9,—Read November 23, 1905.)

(Abstract.)

The material examined was obtained by the author from plants of Welwitschia growing near the German military station at Haikamchab, on the south bank of the Swakop River, 31 miles north-east of the British station at Walfish Bay. Owing to the native rising, it was impossible to carry out the intention of spending some weeks in the country, and keeping plants under constant observation. The results recorded are, therefore, based on the investigations of flowers collected during a hurried visit to the Welwitschia country.

The author deals with the habitat of the plants, and describes the climatal conditions under which they grow. Evidence is adduced in support of the view that Welwitschia is partially, if not entirely, insect-pollinated, and that the processes of fertilisation and maturation of the seed seem to be effected much more rapidly than in other Gymnosperms.

*Male Flowers.*—The author supports Strasburger's view that the male flowers are reduced forms of an originally hermaphrodite structure. The development of microsporangia and microspores is described. The characters of the pollen-grain, in which three nuclei were observed before the dehiscence

of the anthers, are considered to indicate a closer relationship between Welwitschia and Gnetum than between Welwitschia and Ephedra.

*Female Flowers.*—Observations are recorded on the development of the macrosporangia and macrospores; the nature of the prothallial tubes is discussed, and the conclusion is that the true interpretation of the extraordinary behaviour of the fertile end of the Welwitschia prothallus will be founded upon a comparison with the corresponding portion of the embryo-sac of *Gnetum gnemon.*

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# *The Araucarieæ, Recent and Extinct.*

By A. C. Seward, F.R.S., Fellow of Emmanuel College, and Sibille O. Ford, formerly Bathurst Student of Newnham College, Cambridge.

(Received November 13, 1905,—Read December 14, 1905.)

(Abstract.)

The paper includes the following sections:—

I. *Introduction.*—The work was undertaken primarily with a view to ascertain whether the genera *Agathis* and *Araucaria* exhibit any of those features which are often associated with survivals from the past; our aim was to obtain an answer to the question: Do the existing Araucarieæ afford evidence of primitive characters or do they throw light on the phylogeny of the Araucarian phylum?

II. *Distribution.*—A brief account is given of the present distribution of existing species. *Agathis* is for the most part an island type. *Araucaria* is met with in the same area as that occupied by *Agathis*, but occurs also in Chili, S. Brazil, and Bolivia; the most widely spread species—*A. Cunninghamii*—extends over an area 900 miles long in Queensland and New South Wales, and is recorded from New Guinea. Five species of the genus have been described from New Caledonia.

III. *Generic Diagnosis and Synonomy of Species.*—In this section an attempt is made to give a concise account of the more striking characteristics of each species with a list of references to descriptions and records of the several types.

IV. *Seedlings.*—The seedlings described belong exclusively to the genus *Araucaria;* those of *A. Bidwillii* and *A. imbricata*, characterised by the swollen food-storing hypocotyl, are described in detail. In one case the stele

of the young root of *A. Bidwillii* was found to branch into two steles of equal size.

V and VI. *Root and Stem Anatomy.*—A general account is given of the characteristic features of the anatomy of both genera based partly on the work of others and in part on our own investigations.

VII and VIII. *Leaves and Leaf-traces.*—Several types of leaves are described with special reference to the relative abundance of centripetal and centrifugal xylem in the veins. The relation of the leaf-traces to the stem-wood is discussed at length, and some account is given of the behaviour of the traces during the growth in thickness of the stem.

IX. *Reproductive Shoots.*—This section includes an account of the male and female flowers of *Agathis* and *Araucaria*, special attention being paid to the course of the vascular bundles in the sporophylls. We are led to the conclusion that the cone-scales of the female flowers are simple structures homologous with foliage leaves. A description is given of the ovules and embryos of *Araucaria imbricata.*

X. *Fossil Araucarieæ.*—The records of fossil representatives of the two surviving genera are critically examined, and evidence is adduced in support of our contention that the Araucarieæ constitute one of the oldest sections of the Coniferales.

XI. *Phylogenetic Considerations and Conclusion.*—A comparison is made between the Araucarieæ and Lycopodiales; arguments are advanced in favour of the view that this group of Gymnosperms, unlike the Cycadales, was probably derived from Lycopodiaceous ancestors. We draw attention to the various characters in which the Araucarieæ differ from other members of the Coniferales, and suggest the advisability of giving more definite expression to their somewhat isolated position by substituting the designation Araucariales for Araucarieæ.

Our contention is that the general consent which has deservedly been given to the view that the Cycadales and Filicales are intimately connected by descent, may have the effect of inducing an attitude too prone to over-estimate the value of the arguments advanced in support of an extension of the idea of a filicinean ancestry to other sections of the Gymnosperms.

# *On the Distribution of Chlorides in Nerve Cells and Fibres.*

By A. B. Macallum, M.A., M.B., Ph.D., Professor of Physiology, and Miss M. L. Menten, B.A., Assistant-Demonstrator of Physiology, in the University of Toronto.

(Communicated by Professor W. D. Halliburton, F.R.S. Received July 24,—Read December 14, 1905.)

[Plates 2—4.]

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## I.—*Introduction.*

The question of the composition of nerve fibres, and especially of the axon itself, is one which must be solved before a rational explanation of the properties of nerve tissue can be given. The solution of the problem in its ultimate details is possible only when we have at our command reagents and methods which will greatly exceed in sensitiveness those which are accessible to us at present, for such as have been used hitherto comprehend only those of ordinary chemical analysis, and they have not thrown any light on the subject, while those of micro-chemistry, which have been employed up to the present, involve staining with dyes such as one employs in histological technique and treatment with nitrate of silver and osmic acid. The last-named reagent is of service in demonstrating the fat of the medullary sheath, and the nitrate of silver has been employed to show the nodes of Ranvier and the striation known as Frommann's lines, a result which, as will be shown, depends on a micro-chemical reaction, but the dyes hitherto used have revealed little except structure. There are, indeed, the Weigert hæmatoxylin and the Golgi reactions, but the constituents of the sheath and axon responsible for both are unknown. Through these and other histological methods much has been ascertained regarding the structure of nerve fibres and cells, but so far little has been determined which will serve to explain such properties as irritability and conductivity and the nature of the nerve impulse itself.

The results of investigations on the electrical properties of nerve fibres, however, show that the relationship between the electrolytic action of a current on the nerve and its exciting efficiency is a very intimate, practically an indissoluble, one, and in view of this one asks on what constituent the electrolytic effect is exerted.*

The observations of one of us show that in the axons of the normal nerve fibre potassium† in any of its combinations is wholly wanting, and, therefore, the electrolytes present must be those of sodium, calcium, and magnesium compounds, but as the two latter elements are present only in very minute quantities each as compared with sodium in nerve tissue, and as, further, the inorganic phosphates are not found when sought for micro-chemically in the axon, the indications point very distinctly to the occurrence there of chloride of sodium.

The difficulty in demonstrating the occurrence of chlorides in tissues hitherto has been that the one sensitive reaction for chlorides, *i.e.*, the formation of argentic chloride and the reduction of this to subchloride under the action of sunlight, was not distinguishable from that involving compounds of silver formed with proteids and organic compounds generally, which also were supposed to reduce under the action of sunlight. When, therefore, a solution of a silver salt gave a coloured reduction compound on its application to a tissue in sunlight, it was held not to be determinable what was due to chlorides and what to organic compounds.

This difficulty, the senior author has shown,‡ can be overcome if in the reagent itself there is present a quantity of free nitric acid which prevents the formation from phosphates, sulphates, and carbonates, and from all organic compounds of tissues, except creatin and taurin, of coloured reduction combinations of silver, and he found, further, that when albumins, globulins, and gelatins are freed from all traces of chlorides, they do not manifest the slightest capacity to form with silver salts compounds which "reduce" under the action of light.

It became evident, therefore, that a solution of nitrate of silver, containing a quantity of free nitric acid, could be used to determine the occurrence and distribution of chlorides in nerve fibres, and, in consequence, the authors began the observations of which this paper is the outcome.

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* A very interesting discussion of this subject is given in J. S. Macdonald's paper on "The Injury Current of Nerve: The Key to its Physical Structure," 'The Thompson Yates Laboratories Report,' vol. 4, 1902, p. 213.

† Macallum, "On the Distribution of Potassium in Animal and Vegetable Cells," 'Journal of Physiol.,' vol. 32, p. 95, 1905.

‡ Macallum, "On the Nature of the Silver Reaction in Animal and Vegetable Tissues," 'Roy. Soc. Proc.,' vol. B 76, p. 276, 1905.

## II.—*Historical Account of the Literature of the Subject.*

There are no observations, in the literature dealing with nerve fibres, which bear directly on the occurrence of chlorides in these structures. There are incidental, and often indefinite statements, referring to the formation of chloride of silver in the axons of nerves, as, for instance, that of Boveri* when explaining the production of Frommann's lines, but elsewhere in the same work he attributes the silver reaction of nerves not to the presence of any specific substance or compound, but to a precipitate brought about by and in the contact surfaces of tissue elements.

Nitrate of silver has, however, been employed in the study of nerve fibres since 1864, the year in which Frommann made his observations† with that reagent on nerve cells and fibres, and to the results of its use we owe a clearer understanding of the structure of the nerve fibre than we would have without its service. These results were attributed either to physical factors operating on the silver salt and producing a new compound of it which reduced in light, or to an organic compound uniting with the silver to form a combination also affected similarly by light. Ranvier, who made a very extensive use of the reagent, curiously refrains from expressing any view as to the mode of action of the salt, or even as to the possibility of an explanation of its action.

Though the action of the silver nitrate on nerve fibres was unexplained and unknown, yet its use brought out results of importance which, in the light of what we now know, are to be understood as due to the presence of chlorides in the parts affected. Of course the indications which they give of the distribution of the chlorides are very inadequate and very often faulty, but they have furnished some conceptions of the structure of the fibres, and on this account a summary of some of them comes in properly here.

Frommann, on using a solution containing from $\frac{1}{2}$ to 1 grain of silver nitrate to the ounce on nerve fibres of the spinal cord, found on exposure to light a yellow or brown coloration of the axis cylinders. Some had a homogeneous or very finely granular appearance, others appeared distinctly striated transversely, the striæ being placed close to one another. These striæ sometimes obtained throughout the whole visible portion of the fibre, sometimes again only in a portion of the same, the striæ at the terminal parts of the affected extent gradually becoming less distinct. Frequently also axis cylinders were found wholly unaffected, and these would alternate with deeply striated fibres. When the coloration became marked the striation was indistinct. The minutest as well as the broadest fibres exhibited the striæ. The material constituting the matrix of each stria did not appear to be different from that between the coloured bands, and both series varied in breadth even in the same fibre. In parts of certain axis cylinders the striæ were constituted of granules and where striæ were not present the granules were sometimes regularly, sometimes irregularly, arranged with, amongst them, longitudinal fibrillæ.

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* "Beiträge zur Kenntnis der Nervenfasern," 'Abhandl. d. k. Bayer. Akad., Math.-Phys. Cl.,' vol. 15, 1886, p. 423.

† "Zur Silberfärbung der Axencylinder," 'Virchow's Arch.,' vol. 31, 1864, p. 151 ; also "Über die Färbung der Binde und Nervensubstanz des Rückenmarkes durch Argentum nitricum und über die Struktur der Nervenzellen," same volume, p. 129.

Frommann found also that the nerve cells of the spinal cord, on treatment with nitrate of silver, took a light or deep brown, sometimes a grey-blue colour which increased with time. The nucleus in such preparations was as dark as, or lighter than, the cytoplasm, while the nucleolus was as dark as, or darker than, the cell.

The next observer, Grandry,* who studied the action of nitrate of silver on nerve elements, corroborated Frommann in many of the above-mentioned details, and dealt specially with the striæ which are now henceforth known as Frommann's lines. The position of these could be altered by pressure but they could not be destroyed by such means. He claimed that by keeping the preparations for a long time in glycerine the striæ could on compression be completely isolated. There were, in his opinion, two substances in the axis cylinders not mixed or diffused in each other, but wholly distinct, and he compared the structure they present to that found in striated muscle fibre.

Grandry found a very marked striation in the cell body and polar processes of nerve cells of the spinal cord. These striæ varied in diameter even in the same cell, and there was a variation also in their quality, some, the finest, being completely homogeneous, while others were of granular composition. Grandry found at times, as Frommann did, the body of the cell to colour uniformly brown, but the nucleus was unstained. In other cases a portion of the cell body exhibited the striæ while the remainder was uniformly coloured.

This work of Grandry was done in Schwann's† laboratory, and that observer, in discussing Grandry's results, remarked that "it seemed difficult to admit that formations so regular, like the striæ in question, could be artificially produced, if in the living organ there were not a corresponding disposition."

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* "Recherches sur la Structure du Cylindre de l'Axe et des Cellules Nerveuses," 'Bulletins de l'Académie de Bruxelles," 2nd Series, vol. 25, 1868, p. 304; also "De la Structure intime du Cylindre de l'Axe et des Cellules Nerveuses," 'Journ. de l'Anat. et de la Physiol.,' vol. 6, 1869, p. 289.

† 'Bulletin de l'Acad. de Bruxelles,' 2nd Series, vol. 25, 1868, p. 287.

Ranvier* found that in addition to revealing the annular bands of the intersegmental nodes and the portions of the axon in their immediate neighbourhood, the use of the reagent demonstrated the lines of Frommann at the nodes and in their vicinity, the distinctness of the striation gradually lessening with the distance from the nodes. He found also that the axis cylinder at the node exhibited a biconical swelling (renflement biconique) the substance of which blackened with the reagent.

The silver reaction at the nodes is, according to Boveri,† between the two contact surfaces of the cells constituting the neurilemma which meet at that point. Further, a similar reaction obtains in the immediate vicinity of the nodes under the medullary sheath. The silver precipitate, owing to the form of the periaxial space at this point which it occupies, gives the figure called by Ranvier the biconical thickening, and regarded by him as a normal integral portion of the fibre. In Boveri's view the enlargement is a deposit on, not in, the fibre.

A third precipitate which Boveri found obtained wherever the reagent by its action brought the fibrillæ of the axon into contact. This does not occur at definite points, but it is, as a rule, found at the nodes. This precipitate allows the fibrillæ to be recognized except where the periaxial precipitate with which it is in contact is so marked as to obscure them.

Regarding the lines of Frommann, Boveri holds that they are rings about the axon, not in the same, but if the fibrillar structure obtains immediately beneath each, then the precipitate penetrates the fibre and is deposited between the fibrillæ. He rejects the view that the lines are due to a preformed or pre-existing transverse lamination of the axon, because the layers of the silver precipitate decrease in intensity with the distance from the node and at the same time become separated by greater intervals from each other. The most important fact which he found to tell against the view is that discovered by A. Boehm, then an assistant in the histological laboratory at Munich, who placed fine capillary glass tubes, filled with filtered egg albumen, in a 0·5 per cent. solution of nitrate of silver. This diffused into the tube and caused a white precipitate which consisted, in great part, of chloride of silver, disposed not uniformly through the lumen, but in layers, like the lines of Frommann, separated by zones free from precipitate.

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* 'Traité Technique d'Histologie,' Paris, 1875, p. 725; also 'Leçons sur l'Histologie du Système Nerveux,' Paris, 1878, p. 45.

† *Loc. cit.*

As obviously there could not have been any transversely disposed lamination of the albumen in the capillary tube, the result found by Boehm could only be due to physico-chemical causes, and Boveri employs it to explain the production of Frommann's lines. In the lymphatic fluid surrounding the nerve fibrillæ sodium chloride is dissolved, and this gives with the silver solution a precipitate of silver chloride, which in light is reduced and appears as black granules. The medullary sheath acts the part of the wall of the glass capillary tube and prevents the entrance of the reagent except at the nodes or at the torn ends of the fibre, but at these points it slowly diffuses into the axial space. Boveri is inclined to explain the result as due to the penetrating solution losing, through the formation of the precipitate, its silver salt, so that for a time pure water only continues to penetrate until the loss is replaced by silver nitrate diffusing forward, whereupon a new precipitate occurs, but at an advanced point, and water alone again continues to diffuse with the same repetition of result. In this also Boveri finds an explanation for the greater distance separating the lines the more remote they are from the point of entrance, as with the distance from that point a greater interval of the capillary tubule must be traversed before the silver salt can be replaced.

Boveri found further, in small fibres of the sympathetic, a silver precipitate arranged as two parallel bands in the medulla, one on each side of and immediately adjacent to the node of Ranvier. Another precipitate he observed not infrequently in the segments or imbrications of Lanterman.

Joseph,* by treating fresh nerve fibres with a 0·5 per cent. solution of nitrate of silver containing also 5 per cent. free nitric acid, for several hours, and then with solutions of potassium bichromate, found Frommann's lines equally well marked far from the nodes of Ranvier, as at the latter points and over long extents of the fibres. The striæ were formed of granules.

According to Jakimovitch,† who used special methods of applying the nitrate of silver, the striation of the axon is of three kinds, the difference depending on the breadth of the striæ, on the distance between the neighbouring bands, and on the character of the granules composing the striæ. The lines were present in the fibres of a puppy two days old but not in its optic or abductor nerves, though they were found in such in older puppies with the eyes opened. In winter frogs kept in the laboratory the axons were nearly always diffusely granular, not striated, or if striation obtained it was of a feebly marked kind. In such animals, however, after the nerves were cut and then stimulated with induction currents for some minutes, striation was found in a great number of axons.

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* "Über einige Bestandtheile der peripheren markhaltigen Nervenfaser," 'Sitzungsber. k. Preuss. Akad. zu Berlin,' 1888, p. 1321.

† "Sur la Structure du Cylindre-axe et des Cellules Nerveuses," 'Journ. de l'Anat. et de la Physiol.,' 1888, p. 142.

To show the striation the preparations must be perfectly fresh, as those made some time after death reveal few striated fibres, although Jakimovitch found them 24 hours after death in the spinal cord of man and of fishes, but in this case striation was feebly pronounced, while they were absent in an animal dead for six hours, in mice dead from inanition, in frogs slowly poisoned with curare, and in the optic nerves of frogs kept functionally inactive for 8 to 10 days by suturing the eyelids and covering them with a layer of copal varnish. In prolonged chloroform anæsthesia the number of fibres striated were few, as was the case with rabbit dead from paralytic hydrophobia and in birds dead from intoxication.

The striation, Jakimovitch therefore holds, is dependent on functional condition. The striæ are due to the fibrillæ containing, in series, particles which give the silver reaction alternating with others which do not.

He found the striæ also in nerve cells but not in non-medullated fibres such as those of the olfactory nerve, the sympathetic and the nerve fibres of insects and molluscs, which appeared finely granular after the action of the reagent.

That there are, besides nerve cells and fibres, other tissue structures which exhibit, like them, a striation after treatment with silver nitrate, has been shown by Thin,* Reeves† and Flesch‡ in cartilage, and by Rabl§ in the adventitia of blood-vessels, in the connective tissue of the gastric submucosa, in the intercellular material (fluid ?) lying between fat-holding cells, in the hepatic cylinders of the lobules of the human liver, as well as in articular cartilage. The last-named observer obtained these results by treatment of the tissues in question, first with a 1-per-cent. solution of silver nitrate containing also 10 per cent. of free nitric acid, subsequently with a 2- to 5-per-cent. solution of potassium bichromate and followed by exposure to sunlight.

As to the mode of production of these striations Rabl adopts and expands Boveri's explanation.

It may finally be mentioned that some observers have held that the striæ are the expression of periaxial, not intraaxial, structures, and amongst these may be mentioned Rumpf,[*] Morochowetz,[†] Obersteimer,[‡] Lavdowsky[§] and Schiefferdecker,[||] but the deposit which gives the appearance of the striation is, according to Jakimovitch[¶] and Rabl,[**] distributed uniformly throughout the whole transectional area of the axon.

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* "On the Structure of Hyaline Cartilage," 'Quart. Journ. of Micro. Sci.,' vol. 16, 1875, p. 1.

† "The Matrix of Articular Cartilage," 'British Medical Journal,' 1876, vol. 2, p. 616.

‡ "Bemerkungen zur Kritik der Tinctions-Präparate," 'Zeit. für wiss. Mikr.,' vol. 2, 1885, p. 464.

§ "Zur geschichtete Niederschläge bei Behandlung der Gewebe mit Argentum nitricum," 'Sitzungsb. der Wiener Akad., Math-naturw. Cl.,' vol. 102, Abth. 3, 1893, p. 342.

## III.—*Methods of Investigation.*

As already pointed out, it has been shown by one of us that nitrate of silver in the presence of free nitric acid constitutes, in the absence of creatin and taurin, a very sensitive reagent for chlorides, if the preparations are subsequently exposed to light, and that proteids and albuminoids free from chlorides do not give any compound with a silver salt which can be "reduced" in the sunlight.

As a consequence of this discovery the reagent has been used very extensively by us to determine the distribution of the chlorides in the axon. The solution used was a decinormal one of the nitrate, to which 25 c.c. of nitric acid of 1·4 specific gravity (*i.e.*, 60 per cent. strength) were added for every 1000 c.c. of the solution. The reagent, therefore, contained 1·7 per cent. of silver nitrate with 1·5 per cent. of free nitric acid. The distilled water used in making the solution was free from chlorides to the extent that the reagent, when kept in the sunlight for months, did not give the slightest indication of an opalescence, discoloration, precipitate or deposit. This is a point of importance, for the reagent will reveal the presence of chlorine as chlorides when the chlorine is as dilute as 1 in 1,600,000.[††]

The tissues treated were in every case perfectly fresh and, in order that the penetration should be quickly obtained, the parts were carefully and finely teased out on the slide or in the dish before the reagent was added. The latter was allowed to act for a time which in individual cases varied from 1 to 48 hours, and while in the bath of the reagent the preparation was protected from the light. Then it was, when necessary, still further teased out, either quill or glass points being used for this purpose, finally mounted in glycerine of 50 per cent. strength and exposed to the action of bright sunlight till the maximum effect was obtained.

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[*] 'Untersuchungen aus dem Physiol. Inst. zu Heidelberg,' vol. 11, 1882. The reference is given by Rabl.

[†] Rabl, *loc. cit.*

[‡] 'Anleitung beim Studium des Baues der nervösen Centralorgane,' Leipzig und Wien, 1888.

[§] 'Journ. de Médecine Militaire,' 1884–5. The reference is given by Rabl.

[||] "Beiträge zur Kenntniss des Baues der Nervenfasern," 'Arch. für Mikr. Anat.' vol. 30, p. 435.

[¶] *Loc. cit.*

[**] *Loc. cit.*

[††] Macallum, *loc. cit*

In making preparations of the spinal cord a portion of the latter was pressed out between two glass slides to a very thin layer and this, attached to one of the slides, was placed in the reagent for 24 hours, after which it was exposed to light, mounted in glycerine and covered. This was found to be the best way of preparing, with the silver reagent, nerve fibres unprovided with a neurilemma, and it gave some very good preparations of nerve cells showing the distribution of chlorides in them.

Nitrate of silver, when alone in solution, does not penetrate very well, and thus, in the remote parts of even a minutely teased-out preparation, there may be a redistribution of the chlorides before the reagent has reached them by diffusion. When free nitric acid is present, it promotes considerably the diffusion of the nitrate, but, unfortunately, the acid diffuses more readily than the salt, and, in consequence, if the preparations are not carefully teased out, the result may be the same as when the reagent is neutral.

We have used solutions of the reagent containing less than 1 per cent. of the salt with as much as 10 per cent. of the acid. It was found that such a solution gave, in the majority of instances, the most marked preparations of the axons with Frommann's lines, the latter often extending over the axon for the whole, or nearly the whole, extent of the internodal segments of the fibre. This solution, however, has the disadvantage of redistributing chlorides before the silver nitrate reaches them. Instances of this were found in nerve fibres of the sciatic of the frog and rat in which a portion of the chlorides diffused, in the parts remote from the nodes, from the axon into the medullary sheath, and were then precipitated by the reagent. It was observed also that the reagent of this composition gave very often in ordinary connective tissues, as, for instance, in those of the pancreas of the guinea-pig, in the adventitia of arterioles, and in the gastric mucosa of the frog, a striation in every respect like that found in the axons, and exactly similar to that described by Rabl in such structures. Such striations are artefacts, and, therefore, a high degree of concentration of acid in the reagent tends to show a distribution of the chlorides that does not obtain in the living fresh structures.

The best results are so obtained, as our experience shows, with a decinormal solution of the salt containing 1·5 per cent. nitric acid. When the preparations had been acted on by it for the required time, they were mounted, without removing the excess of the reagent, in 50 per cent. glycerine. The advantage in this is that, as in the photographic plate, an excess of nitrate of silver acts as a "sensitizer," that is, it combines with the chlorine liberated by the reducing action of the sunlight from the already formed chloride of silver, and this addition to the precipitate, under the influence of the light, itself is reduced, thus rendering more pronounced the original reaction. This serves to demonstrate the presence of infinitesimal traces of chlorides, and, consequently, when we find, after the fullest precautions have been taken, that a tissue element or a cellular constituent gives no silver reaction, we can be certain that haloids are not present, in other words, such structures are absolutely free from them.

It may be objected that, at most, only 8 per cent. of the chloride is converted into the subchloride, and, therefore, if the quantity of the chloride formed should be very minute, that of the subchloride resulting being not more than one-twelfth of the other, might escape observation, or be invisible, while the structures under consideration could not be considered to be free from chlorides. This may be met by pointing out that, if we add nitrate of silver to a solution of sodium chloride corresponding to one part of chlorine in 1,000,000, the opalescence resulting is distinct, and, after the action of sunlight, the reduction is observable. Here one part of chlorine in 12,000,000 at least of water serves to demonstrate not only its own presence but also that of the other 11 parts.

On this account the reaction is the most sensitive, the most delicate one that is to be found in micro-chemistry. When it is not obtained in a tissue or cellular element, one may safely conclude that chlorides are wholly absent from it.

There is another reagent of which we made some use, and which we found serviceable. This was a solution of mercurous nitrate. When a quantity of this is added to a solution of a chloride, there results, as is well known, a white precipitate of mercurous chloride, whose solubility is 3·1 parts in 1,000,000 of water at 18° C., while the chloride of silver is soluble to the extent of 1·7 parts in the same quantity of water at the same temperature.* The mercurous chloride is, therefore, slightly more soluble than the silver chloride, and the mercurous reagent is consequently less sensitive than the other as a precipitant for chlorides, but against this may be placed the fact that it is possible in tissues to reveal the presence of *all* the mercurous chloride formed, while the presence of the whole of the chloride of silver precipitated is demonstrated by only one-twelfth of its quantity developed as subchloride.

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* These results were obtained by calculation from the electrical conductivity of $AgCl + Aq$ and $Hg_2Cl_2 + Aq$, by Kohlrausch and Rose ('Zeit. für Physik. Chem.,' vol. 12, p. 241). Halleman (*ibid.*, p. 132) found 1 part of AgCl soluble in 715,800 parts of water at 13°·8 C., and in 384,100 at 26°·5 C. Stas, on the other hand, states ('Untersuchungen über Chem. Proportionen,' Transl. by Aronstein, pp. 46 and 47) that 1/20, and even 1/100 milligramme of silver can be demonstrated in 1 litre of water by the addition of sodium chloride.

The preparation of the reagent is that described by Fresenius:[*] one part of pure nitric acid of 1·2 specific gravity is poured on one part of pure mercury, and the containing vessel is allowed to stand 24 hours in a cool place. The crystals formed are separated from the undissolved mercury and the mother liquor, and, after trituration in a mortar, dissolved in water mixed with one-sixteenth part of nitric acid. The solution, which must be saturated, is filtered, and the filtrate is kept in contact with metallic mercury. When the reagent is to be used, one part of the solution is diluted with three parts of water.

The reagent is applied to the teased-out nerve fibres, and left in contact with them for half an hour, after which they are washed in distilled water, frequently changed for about two to three hours, and finally mounted in a mixture of 50 per cent. glycerine and ammonium sulphide, which latter converts the white mercurous chloride precipitate into black mercuric sulphide. If it is properly washed with water to remove the precipitant, the final reaction should indicate the distribution of the chlorides.

The results obtained by the use of this reagent corroborated the conclusions drawn from the results of the use of the nitrate of silver. As a reagent for the detection of chlorides it is not a ready penetrant of tissues, and this cannot be overcome by the addition of an acid, as the latter is apt to convert it into the mercuric salt, which is not a precipitant for chlorides.

## IV.—*The Results.*

A. *In Nerve Fibre.*—If one places a teased-out portion of the sciatic nerve of a frog, guinea-pig, or rat in the silver reagent for about half an hour, and then, without further treatment, in the sunlight, inspection under the microscope reveals the occurrence in the fibres of the appearances known as the crosses of Ranvier, structures which are due to a deposit of silver chloride in the annular ring of the node and in the axon in the immediate vicinity of the ring, the silver chloride on reduction having a dark brown, or sepia, or even black colour. There is no evidence in such preparations of chlorides in other parts of the axons. That the crosses are due to the precipitation at those points of chlorides is shown by the fact that the reagents which dissolve silver chloride also dissolve this deposit before it is reduced by light. For example, calcium chloride, magnesium chloride, sodium thiosulphate, potassium cyanide and potassium and sodium chlorides, each in 5-per-cent. solution, dissolves out the deposits, beginning first with the annular ring, and proceeding inwards along the axon. Fig. 2, Plate 2, represents preparations in which the earlier stages in this solution have already taken place, and in consequence the precipitate in the immediate vicinity of the node has been removed by the potassium cyanide solution.

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[*] 'Manual of Qualitative Chemical Analysis,' American Edition, 1898, p. 111.

When the preparation was first of all put into cane sugar solution of 5·60 per cent. strength, or into a 1·09-per-cent. solution of potassium nitrate,* the shape and general appearance of the fibres were on the whole maintained, but subsequent treatment with the silver reagent gave no silver reaction at the nodes. These solutions dissolve out of the nerve fibres, or at least from the neighbourhood of their nodes, the salts they contain, and the sugar or the nitrate diffuses in, with the result that no precipitate of chloride of silver occurs. Also when the fresh preparations are placed for even a couple of minutes in distilled water, there is a considerable diminution in the amount of the silver precipitate at the nodes, though Frommann's lines may be found for a short distance on each side of any one of them. When their stay in distilled water covers as much as 15 minutes, the silver precipitate does not appear at the nodes or elsewhere.

One may admit that these solutions dissolve out other constituents in addition to chlorides, and consequently the absence of a silver reaction in such a case does not of itself alone point to the chlorides as the necessary factor in the precipitation, but taken in conjunction with the results of the treatment of the unreduced precipitate with solutions of potassium cyanide, sodium thiosulphate, the chlorides of calcium, magnesium, sodium, and potassium, as well as with dilute ammonia, all of which dissolve more or less readily silver chloride, one can but conclude that the precipitate which forms at the nodes is that of chloride of silver. There is, further, the fact that there are no compounds, either inorganic or organic, except the chlorides and creatin and taurin, in the physiological economy which, in the presence of free nitric acid, give with silver salts precipitates which darken in the sunlight, and it is from data supplied by analyses of nerve tissue impossible to regard the silver precipitate of nerve fibres as in part due to either creatin or taurin, or both.

This conclusion is further fortified by the results of treatment of nerve fibres with the mercurous nitrate reagent, followed, as described in the section on methods, by the application of ammonium sulphide. The reaction depends on the formation of the highly insoluble mercurous chloride and the demonstration of the presence of this precipitate by its conversion into the black mercuric sulphide. When care is taken in using this method, the results, which will be more fully described below, are curiously like those which the silver reagent furnishes (figs. 10 and 11, Plate 3).

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* These solutions are approximately isotonic with 0·64-per-cent. solution of sodium chloride.

That the darkened silver precipitate is not metallic silver is shown by the fact that it is soluble in solutions of potassium cyanide, but insoluble in dilute nitric acid, which latter reagent readily dissolves metallic silver. These results indicate that the reduced silver compound in the nerve fibre reacts in the same manner as does the subchloride of silver when similarly treated in the test tube.

When nerve fibres are left for a longer time than half an hour, for example, 24 hours, in the silver reagent, the reaction is found to cover a greater extent of the fibre, and it may occasionally happen that it extends over nearly all an internodal segment, leaving only a slight extent of the axon, midway between two nodes, unaffected. Here one may see the lines of Frommann very distinct, and one finds invariably also that each of the dark striæ increases in superficial area with the distance from the node, but at the same time they diminish in the intensity of their tint, and are separated from each other by wider interstriate zones. This result would appear to be due to two causes, one of which is that, remote from the nodes of Ranvier, the axon swells to several times the diameter of the axon at the node, and the other that the reagent, which mainly attacks the axon through the node, becomes more dilute as it penetrates. This dilution of the reagent, which affects only the silver salt, not the acid, robs it of its fixing power, and thus promotes its distending effect on the axons. Sometimes there is found the further result, represented in fig. 1, *a*, where the chlorides are diffused from the swollen axon into the immediately adjacent medulla, and consequently Frommann's lines fail to appear there. This result may be, and often is, much more pronounced in preparations treated with a decinormal solution of silver nitrate containing from 5 to 10 per cent. of free nitric acid. An example of this is represented in fig. 8, where the medulla of the parts remote from the nodes is shown impregnated throughout with chlorides diffused from the axon.

It is, therefore, at the nodes and in their immediate vicinity that one obtains always, or nearly always, the best silver chloride reaction, regard being had to the character and disposition of the precipitate, and to the proper fixation of the axon, and it is also in the neighbourhood of the nodes that one finds, after treatment of nerve fibre with the silver reagent, the most typical examples of Frommann's lines.

To get the reaction extend as far as possible along the axon, one must treat the nerve fibres with a decinormal solution of silver nitrate in which the strength of the acid has been increased to 10 per cent. Acted on for four or five hours many fibres show the typical Frommann striation to extend over considerable extents of their axons. There are, as already pointed out, in such preparations fibres which show very considerable alterations in the disposition of the chlorides, and this is a serious objection to the employment of a solution of the reagent in which the nitric acid is so highly concentrated. If the reagent of this strength diffuses into the fibre properly, the result should be, and usually is, a striking demonstration of Frommann's lines over a considerable extent of many of the affected axons. On the other hand, a diffuse silver reaction in the axons of fibres of the same preparation is, in the majority of cases at least, the result of the failure of the reagent to penetrate normally, and the diffuse reaction itself is very often an indication of an artificially altered distribution of the chlorides of the axon.

It is otherwise with nerve fibres which, like those of the spinal cord or of the optic nerve, are unprovided with neurilemmal sheaths, for these may, and oftenest do, show a uniformly diffused reaction for chloride of silver extending over long stretches of the axons and wholly confined to them. These axons are not swollen or altered in any morphological aspect, and everything indicates that the reaction they give is not, as in the case of fibres provided with neurilemmata, due to imperfect penetration, but rather to rapid diffusion into the axons and the immediate precipitation of the chlorides before these can be redistributed.

Such preparations may be obtained by pressing portions of the spinal cord between two slides to very thin layers, and treating them at once and for 24 hours with either the ordinary reagent or that in which the acid constituent is of 10-per-cent. strength.

Many of the fibres in such cases are easily and completely interrupted at points by the pressure to which they are subjected, and in consequence one may find amongst them portions of axons which reveal a Frommann striation as pronounced as any that may be found in fibres provided with neurilemmata, and these lie side by side with greater lengths of uninterrupted nerve fibres which show a diffuse silver reaction confined to unswollen and normal axons.

The explanation for the differences in the silver reaction between fibres from the spinal cord on the one hand and those from ordinary nerves on the other must be referred to the absence or presence of the neurilemma. Ordinary nerve fibres are not readily penetrated by the silver reagent except at the nodes, and even when the nitric acid in the reagent is of 10-per-cent. strength, the point of entrance is still, in great part, if not wholly, the node, whereas in preparations from the spinal cord, the medullated fibres of which have no neurilemmal sheaths, the noteworthy feature is the penetration, by even the weaker reagent, of long sections of such fibres in as short a period as an hour, an interval of action on nerve fibre with neurilemmal sheaths which would allow only of the demonstration of little more than sufficient to bring out the crosses of Ranvier.

In support of the view that the neurilemma acts as a very considerable barrier to the penetration of nitrate of silver, the results of observations on nerve fibres of invertebrates may be detailed. In the decapod crustacea (lobster and crayfish) the nerve fibres of the ganglionic cord are ensheathed each in membranes which, except for the nuclei observed here and there on their course, are homogeneous and vary very considerably in thickness with the fibres which they cover, being 0·5 to 2 $\mu$ in the extraordinarily thick fibres (100 to 150 $\mu$) and discernible with difficulty in those which are very narrow (2 to 5 $\mu$).* This sheath is, therefore, morphologically different from the neurilemma of vertebrate nerve fibres, but it certainly exercises the functions of that structure.

When the teased-out fresh fibres of the ganglionic chain of the lobster or crayfish are treated with the acid nitrate of silver solution for 24 hours, the reagent penetrates the sheaths of the larger fibres only slowly, and gives to their contents a diffuse chloride reaction, usually a feeble one, but varying according to the degree of penetration. The penetration of the smaller fibres is always more pronounced, owing to the greater tenuity of the membrane, and, in consequence, one gets reactions, like that represented in fig. 9, *a*, limited to the contents in which, besides the diffused condition of the chloride precipitate, there is also a finely granular deposit of the latter.

When the reagent, instead of entering the nerve fibre through the intact sheath, gains access to the axial contents by the open ends of a torn or interrupted membrane, the result is a Frommann striation. This has been seen by us only very rarely in the case of the larger fibres, but frequently in the smaller ones, long segments of which exhibited the peculiarity. The striation found was typical (fig. 9, *b*), and was often seen in fibres immediately adjacent to others whose sheaths were intact and in which the reaction was a uniformly diffuse one. This is, we believe, the first instance observed of the production of the striæ in the nerve fibres of invertebrates, and the failure of Jakimovitch and others to obtain such striæ in their preparations may have been due to the possibility that they did not tease out their preparations so as to allow the reagent to penetrate sufficiently the parts, or that they did not make sufficiently extensive observations on their material.

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* Emil Yung, "Recherches sur la Structure Intime et les Fonctions du Système Nerveux Central chez les Crustacés Decapodes," 'Arch. de Zool. Expér.,' vol. 7, p. 401, 1878.

That the neurilemma in vertebrate nerve fibres is not the only structure preventing the entrance of the reagent is shown by the fact that even in the fibres of the lateral columns of the cord (guinea-pig, rat, and rabbit) the reagent takes time to penetrate. This must be due to the medulla, for in the pressed-out preparations the short portions of fibres with free ends in many cases show Frommann's lines, an indication that the reagent penetrated at the open free ends, and that where a more or less long fibre, not cut by the pressure into portions, exhibited the striation of its axon, it was caused by an injury at some point on its course, permitting the reagent to diffuse into the fibre there more readily than through the intact medulla. It is to be noted that owing to the absence of the neurilemmal sheath, the medullated fibres of the spinal cord are much more fragile than are those provided with Schwann's sheath, and it is owing to this that pressure will give isolated short segments in the former readily and in the latter only with difficulty and then rarely. It is these segments, as a rule, that in the spinal cord, as already stated, show most often the striation. Such segments may be compared to the segments or short portions of capillary glass tubes filled with egg albumen which, as will be described below, give, when placed in a bath of the silver reagent, a striation like in every respect that found in silver preparations of medullated fibre.

One may in a similar manner explain the results obtained by applying the mercurous nitrate reagent to nerve fibres to demonstrate the distribution of the chlorides. As already pointed out, this reagent is but little less sensitive a test for the chlorides than is the nitrate of silver, and, when it is carefully applied, it gives quite all the results that the other does. It, for example, brings out the crosses of Ranvier (figs. 10, *a*—*d*). In the parts of the axon remote from the nodes it demonstrates the chlorides, but the mercurous chloride precipitate is often not uniformly distributed in the axon (figs. 12—15), and the latter is frequently curiously altered in form and character (fig. 14). Sometimes the precipitate is on the surface of the axon and between it and the medullary sheath. As a rule, it is rarely found to occur for more than a short distance on each side of the node, and here it may give the Frommann striation. In fibres which, as is rarely the case, the striation is in parts of the axon remote from the nodes (fig. 11, *a*), it would appear as if the reagent penetrated the fibre by the clefts of Lanterman (fig. 13). Such, and a number of other like results, seem to indicate that there are weak points in the medullated fibre provided with a neurilemma, but that, on the whole, the latter is a bar to the direct penetration to the axon by the reagent.

Quite otherwise was the result of treatment of teased-out preparations of the spinal cord (rat) with the reagent. In such preparations there were a large number of medullated fibres which exhibited, over long stretches of their axons, a striation quite as marked and as characteristic as one may obtain in pressed-out preparations of the cord treated with the nitrate of silver reagent. Such a striation as obtained by the mercurous nitrate reagent is represented in fig. 16. Nothing approaching this has been obtained with the same reagent in nerve fibres having a neurilemma. On the other hand, this reagent can pass through the medulla of the fibres of the cord, and give, as did the nitrate of silver, a diffuse reaction for chlorides.

There can, therefore, be no doubt that the axon is rich in chlorides, but that these cannot be demonstrated readily therein, except at the nodes, in fibres provided with a neurilemmal sheath, and the difficulty is due, though not wholly, to the neurilemma. When this is absent one may obtain such a demonstration more or less readily, and the result appears in the form of the Frommann striation or in a diffuse reaction throughout the axon.

It is now a question whether the striation or the diffuse reaction indicates the true distribution of the chlorides in the fibre. That the striation cannot represent it seems to be indicated by the fact that the striæ which are remote from a node may be and are usually less distinct, broader and wider than those in the neighbourhood of a node, and that there are degrees of transition from the distinct narrow to the less deeply stained broad ones. Grandry, Joseph and Jakimovitch hold that it demonstrates a pre-existent arrangement of substance in the axon, and the last named would explain their presence as a necessary factor in the functional activity of nerve fibres, while Boveri and Rabl regard the striation as not due to a pre-existent disposition of the substance of the axon, the former explaining the striation as a result of physical causes.

A good deal of light has been thrown on the causation of this striation by the discovery of Boehm* that solutions of egg-albumen in capillary glass tubes, when placed in solutions of nitrate of silver, show the silver chloride as a precipitate to appear as striæ transversely placed in the lumen, and more recently by the observations of Liesegang† on the diffusion of silver nitrate in films or columns of gelatine impregnated with potassium bichromate. When a drop of nitrate of silver solution is placed on a glass plate faced with a film of gelatine impregnated with the bichromate salt, the silver salt diffuses slowly and in an ever-growing circular area concentric with the drop, and, as it diffuses, it forms a precipitate in the gelatine of silver chromate which, however, is not deposited uniformly, but in rings, all concentric also, each separated from its neighbour on both sides by clear zones free, or almost free, from silver chromate. These zones increase in width from the centre to the periphery. When the bichromate-holding gelatine is in open capillary tubes the silver salt penetrates, and as it diffuses in silver chromate-holding zones, develops alternately with silver chromate-free bands, and the latter increase in width with the distance from the point of penetration, *i.e.*, the open end of the capillary tube.

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* Boveri, *loc. cit.*, gives the only account accessible of Boehm's discovery.

† 'Chemische Reaktionen in Gallerten,' Düsseldorf, 1898; also Liesegang's 'Photo. Archiv,' 1896, p. 321.

The phenomenon found by Boehm was explained by Boveri as indicated in the section on the literature. Ostwald* has applied to Liesegang's rings the explanation that in the diffusion of the silver salt and the consequent formation of silver chromate, the metastable and labile conditions of solutions of the latter salt alternately prevail, the metastable stage as it is developing into the highest degree of supersaturation, *i.e.*, the labile condition, diffusing through an extent of the gelatine film or of the gelatine column that is to constitute an interstriate zone. When the critical concentration in the advancing solution is reached, then the precipitation begins and continues, thus forming a stria. The precipitation brings the solution back to the metastable condition, then another development into the labile condition obtains and as before a new interstriate zone and a new stria result. This process is repeated indefinitely so long as the diffusion occurs, but as the silver salt becomes more and more dilute, critical concentration is later and later attained, and in consequence the new striæ are separated from each other by broader and broader interstriate intervals. Also, although this is not included in Ostwald's explanation, the striæ themselves must, for the same reasons, become broader but less pronounced in density.

Ostwald's explanation is accepted by Morse and Pierce,† who, working on the rate of diffusion in capillary tubes filled with chromate-holding gelatine when placed in silver nitrate solutions, found $x/\sqrt{t}$ is a constant. The phenomenon has also been studied by Hausmann,‡ who determined that the rate of formation of the precipitate is nearly proportional to the rate of diffusion of the ions of the salts concerned, and in consequence it has been suggested that the rate of precipitation be taken as a measure of the velocity of migration of the ions, and Traube, in his comments* on the same paper, remarks that the formation of the vesicular and reticular structures in cell protoplasm are due to the same process that evolves the rings and striæ described by Liesegang.

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* 'Zeit. für physik. Chem.,' vol. 22, p. 302, 1897, and vol. 23, p. 365, 1898; also 'Lehrbuch der allgem. Chemie,' 2 Aufl., vol. 2, p. 778.

† "Diffusion und Übersättigung in Gelatine," 'Zeit. für physik. Chem.,' vol. 45, p. 589, 1903.

‡ "Über Niederschlagebildungen in Gallerten," 'Zeit. für anorg. Chem.,' vol. 40, p. 110, 1904.

In order to study the relation of these striæ to those found by Frommann in medullated nerve fibres, we prepared capillary tubes with filtered egg albumen, cut them into short pieces 10 to 15 mm. in length, and placed them in the N/10 nitrate of silver solution containing 1·5 per cent. of free nitric acid. They were observed under the microscope with powers magnifying from 90 to 590 diameters, and the progress of the reaction, exposed as the tubes were to a bright sunlight, could be followed from point to point readily because of the reduction of the silver chloride. This reaction advanced from both ends of each section but it usually ceased before the middle of the tube was reached.

In every case the striæ appeared pronounced. The appearances which they gave are represented in figs. 20 and 21, *a* being the part of the column nearest the entrance of the tubes. In both it is seen that the striæ cease at a point beyond which only a general and diffuse precipitation of silver chloride is found. This is not always the case, for one finds in some tubes also where the striæ cease, so likewise does the precipitation. With a higher magnification it is shown that the interstriate zones contain a fine precipitate whose occurrence may be overlooked. It is to be noticed also that the borders of the striæ are not linearly or sharply defined, for the granules are often scattered and sparse at the edge of the stria, but in its centre they are densely aggregated. It is found also that there usually is a gradual broadening of both the striæ and the interstriate zones as one follows the column inwards, and the striæ become at the same time less and less distinct until, as represented in fig. 21, they are lost in the ordinary precipitate. Sometimes an interval of unstriated material, showing an irregularly disposed precipitate, interrupts the striated column at one end, and sometimes also the interruption may be very short, and of such an appearance as to suggest a complete fusion of several striæ. Now and then one may find that the interruption is intensely coloured, this apparently depending on a greater concentration of sodium chloride in the albumen at the point than elsewhere in the tube. The striæ on either side of the interrupting area may be very dissimilar in breadth and not belong to the same series.

In order to determine whether concentration of the chlorides in the albumen would influence in any way the character of the striation, we added to portions of the albumen enough sodium chloride to make the concentration of that salt double, treble, quadruple, and so on, of the original amount of chlorides present,* and capillary tubes filled with such albumen solutions were prepared. It was found that concentrations beyond 3·168 per cent. of sodium chloride did not give striations, while they were infrequently obtained in concentrations between this limit and that of 2·016 per cent. and then only near the ends of the capillary tubes.† At the very entrance the striæ, if definable, were so with difficulty, and the albumen column at and near this point manifested more often a dense, uniform reaction.

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* *Ibid.*, p. 145.

In order to determine if gelatine gives different results, tubes filled with commercial gelatine‡ were treated as in the case of albumen, and it was found that the striæ and interstriate zones were always broader, while interruptions were very frequent in the striate sections of the tubes. Even when the percentage of chlorine was made equivalent to that of egg-albumen by the addition of a sufficient quantity of sodium chloride, the striæ were always broader, as were also interstriate zones, than in egg-albumen; figs. 22 and 25 illustrate this difference, the former representing an albumen preparation, the latter a gelatine one.

The reason for this difference is that gelatine is a solvent to a certain extent for argentic and argentous chloride and in consequence the critical concentration, the labile condition, can be attained only after a longer interval of time than that required in the case of albumen, during which the silver salt is diffusing onward in the column. This alone would entail in each case a very broad interstriate zone and also a broad zone of precipitation.

The Frommann striation in nerve fibres is in no whit different from that found in capillary tubes. As already pointed out, the striæ in nerve fibres broaden with the distance from the node of Ranvier, while in the neighbourhood of the latter they are almost in contact with one another and give the axon here a uniformly dense reaction. With the distance also each stria

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* The amount of chlorine as chlorides in the white of egg, as calculated from the analyses of Poleck ('Pogg. Annal.,' vol. 79, p. 155), and Weber (*ibid.*, vol. 71, p. 91), ranges from 0·165 to 0·1848 per cent. The mean between the two extremes, 0·1749 per cent., corresponds to 0·288 per cent. of sodium chloride. To quantities of the original albumen enough sodium chloride was added to make a series of percentages from 0·576, 0·864, 1·152 up to the twelfth multiple, *i.e.*, 3·456.

† Macdonald ("On the Injury Current of Nerve: the Key to its Physical Structure," 'Thompson-Yates Laboratories Report,' vol. 4, 1902, p. 213), on the basis of the electrical conductivity of nerve fibres, has estimated it to be that of a 2·6-per-cent. solution of KCl. The chlorine of such a solution would correspond to that of a 2·04-per-cent. solution of NaCl.

‡ The quantity of chlorine in specimens of the commercial gelatine we used was determined by us and found to be 0·41 per cent. In a 10-per-cent. solution of gelatine the chlorine would be only 0·04 per cent.

becomes more distinctly resolvable into granules. Further, there are now and then in the striated portion of the axon short segments where the deposit is a uniform one, and on either side of one of these segments the striæ differ in their breadth so much as to suggest that they do not belong to the same series.

Of all these points one finds illustrations also in glass capillary tubes. Further, the fusion of several striæ referred to as occurring in glass tubes occurs in the axons, and has been described under the term biconical swelling ("renflement biconique" of Ranvier). The enlargement cannot, of course, take place in a rigid tube while the medullary sheath and the neurilemma are to a certain extent elastic and permit such alterations in the thickness of the axon as the enlargements referred to. Boveri held that the enlargements are really an extra axial or periaxial deposits of silver at the node, but the fact that we can obtain such enlargements in the medullated fibres of the cord (rat, guinea-pig and frog) where there are no nodes, does not lend any support to this view.

There is but one feature in the Frommann striation which we have not found in capillary tubes, and it does not occur very frequently. This is seen in a division of a single striation into two imperfectly separated halves (fig. 6, *b*). This may be explained as due to a local condition of the nerve fibre like or similar to that which in capillary tubes may result in producing an interruption of the striated column.

In view of all these facts, the Frommann striation must not be regarded as an indication of the disposition of the chlorides in transversely arranged zones in the nerve fibre *intra vitam*, but it is rather the result of the operation of physical processes which in capillary tubes produce the Boehm (or Liesegang) phenomenon.

The medullary sheath is in the great majority of the nerve fibres free from chlorides, but in the remainder a reaction for chlorides may be obtained at points along the course of the sheath, as in the preparation represented in fig. 18, which is of interest in view of the fact that a similar distribution of a potassium salt has been observed occasionally,* and it would indicate that chloride of potassium is present at these points. It is, however, more frequently in the imbrications of Lanterman than in other parts of the medullary sheath that one obtains the reactions for chlorides. One finds now and then all the imbrications in a long extent of the fibre revealed by the chloride reactions (fig. 19), and so fully as to bring into prominence the funnel-shaped outlines of these structures (fig. 10, *d*). In these, also, the potassium reaction was occasionally obtained and, therefore, a part at least of the quantity of the chlorine present as chlorides must occur as potassium chloride.

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* Macallum, 'Journ. of Physiol.,' vol. 32, p. 95.

The fact that this salt occasionally occurs in the imbrications, is an indication that these are the weak points in the sheath through which the salts of the lymph bathing the nerve fibres can penetrate to the axon. Indeed, as already stated, an imbrication may be the point of entrance for the reagent to bring out the Frommann striation (fig. 13, *a*). The double character of each of the imbrication funnels seems to show that the chlorides do not obtain so much in the cavity of the clefts of the imbrications as in their walls.

The absence of chlorides from the rest of the medullary sheath in the normal nerve fibre is noteworthy, and can be interpreted as signifying that the medulla is a very effective barrier to the passage, on the one hand, of chlorides from without the nerve fibre and, on the other, of the haloid salt from the deeply charged axon into the contiguous fat-holding neurokeratin network. The retention of the chloride in the axon must in very large part, if not wholly, be due to the property of the sheath.

B. *In Nerve Cells.*—Frommann, Grandry, and Jakimovitch found that nerve cells give a reaction with silver salts like that which may be obtained in the nerve fibre, but Frommann found that it was a diffuse one in the cytoplasm, more or less implicating the nucleus and the nucleolus, while the two last named observers obtained in their preparations a transverse striation of the nerve cells like, in every respect, that occurring in the axons, and apparently constituting a continuation of it.

The occurrence of a striation has been observed by us in a few of the nerve cells from the anterior horn of grey matter of the spinal cord of the frog and guinea-pig, the striation affecting, in the majority of instances observed, only a portion of the cell, and appearing to be an extension of the striation from one of the polar processes of the cell. The nucleus was in not one instance affected, and when it appeared to be, it was found that the result was really due to the colour reaction of the cytoplasm above or under the plane of the nucleus.

These instances of striation of the nerve cell were exceptional cases. In the pressed-out preparations of the spinal cord treated with the acid nitrate of silver solution for 24 hours, when the nerve cells were affected they usually showed a brownish-yellow reaction of the cytoplasm, but an absolutely uncoloured nucleus and nucleolus. The reaction was a diffuse one, but sometimes so far particularised as to give the appearance of a longitudinal striation, suggesting a slight degree of fibrillation, yet in no case was it marked enough to indicate that it was anything more than an artefact. A more pronounced degree of fibrillation was found by Frommann and Grandry, but their results in this respect may be explained as due to the same cause, for they left a weak solution of the reagent in contact with the cells for a time sufficient to bring about alterations in structure.

Nerve cells are very difficult of penetration by the acid reagent, and in consequence those of the sympathetic and spinal ganglia in vertebrates, and of the ganglionic cord in crustacea (lobster and crayfish), only rarely give a reaction extending throughout all the cytoplasm, this being limited usually to the more peripheral portions of the cell protoplasm. So deep, however, was this peripheral reaction in certain cases, that the interior of the cell, including the nucleus, was obscured. When the whole of the cytoplasm was uniformly affected, the colour was less pronounced, and the nucleus with its silver white appearance was visible. The deep reaction limited to the periphery in some of the cells, and the lighter diffuse reaction in others, seem to suggest that when the reagent does not readily penetrate, the chloride present diffuses out to meet the silver salt and, accordingly, to give in the outer zone of the cytoplasm a silver chloride deposit more abundant than that which would be there if there were no diffusion and redistribution of the chloride. In the pressed-out preparations of the cord of the guinea-pig and frog, the reagent gained access at once to the cytoplasm of many of the cells because the greater part of the surface of each was thus exposed to the solution, but in not one instance did the reaction advance beyond the brownish-yellow colour.

The light reaction of the cytoplasm, considered with the fact that the latter is usually voluminous, may be held to signify that the protoplasm of nerve cells is less rich in the chloride than is the axon which arises from it.

In several preparations we found such a disposition of the chloride reaction in the cytoplasm as to make us question whether the Nissl granules contain any of the chloride present in the cytoplasm generally. The reagent does not bring out these granules usually, and when they are seen, it is through the cytoplasm coloured by the reaction. They thus appear affected by the reaction, and it is consequently difficult to say whether they are free from chlorides or not.

That the nucleus of the nerve cell is free from chloride compounds is not true only of these cells, for the senior author's observations go to show that the nuclei of all cells, animal and vegetable, which are in a normal condition are absolutely free from chlorides.

### V.—*General Remarks.*

The observations detailed in the foregoing pages make it possible, at least to a certain extent, to understand nerve conductivity, and perhaps also nerve irritability.

The occurrence of chlorine in the form of chloride or chlorides in the axon, uniformly distributed throughout its extent, may now be regarded as established. The concentration in the axon also is greater than that in the fluid lymph bathing the fibre, for the silver reaction in the elements external to the fibre does not at all approach in intensity that which may be obtained in the axon. As lymph contains not more than 0·62 per cent. sodium chloride, the concentration in the axon must considerably exceed that.

If the chlorine is present as more than one chloride compound, then it must be in combination with sodium, calcium, and magnesium, potassium being excluded.* If only one chloride obtains in the axon, it is inferentially that of sodium.

The chloride or chlorides are, in part at least, in solution in the material constituting the axon, and thus obtains the electrolytic condition which would explain the conductivity of the axon, the propagation of the nerve impulse itself, and apparently also the excitable efficiency of the nerve fibre.

That all of the chloride or chlorides cannot be in the ordinary state of solution would seem to follow from the facts regarding the velocity of the nerve impulse. If the latter is, fundamentally, an electrical change in the process of being propagated along the axon, it should, provided the chloride or chlorides are in a simple state of solution, have a velocity enormously greater than its maximum has been ascertained to be, that is, 64 metres per second.

On the assumption that the nerve impulse is, fundamentally, electrical, such a diminution in the velocity postulates a disposition of the chloride or chlorides in the axon very different from that in a simple aqueous solution of them. This difference may be attributed to the condition of the colloid material in the axon.

A "solution" of colloid matter, at least of the organic sort, has, as is well known, properties very unlike those of a solution of a simple inorganic compound, sodium chloride or potassium sulphate, for example, properties which specially distinguish it from solutions of inorganic compounds in respect to osmotic pressure, electrical conductivity, cryoscopy and the effect exercised on transmitted light. These properties, and particularly the facts obtained by the use of the ultramicroscopic apparatus on "solutions" of colloids, compel, as it were, the conclusion that the necessary feature of such "solutions" is a state of suspension of particles of a magnitude beyond the limits of microscopic vision.* It is not necessary to discuss the cause of such a state of suspension or why it is indefinitely maintained without any trace of sedimentation, as the force or forces concerned, whether electrical, as Bredig, Hardy and Billitzer hold, or otherwise, do not immediately affect the question of the physical division of the colloid in the fluid, and we have now only to deal with the problem of the structural character of the particles in suspension. How these particles in their contact relations with the molecules of water differ from inorganic compounds in the same situation is not determined, but the dominant view is that the colloid particles are not single molecules but aggregates of such molecules and, of course, this excludes the possibility of an intimate association of the molecules with those of water.

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* Macallum, 'Journ. of Physiol.,' vol. 32, p. 1, 1905. *Note.*—Macdonald, in a recent contribution ("The Structure and Function of Nerve Fibres," 'Roy. Soc. Proc.,' B, vol. 76, p. 322, 1905), holds that potassium is present in a concealed, or "masked," condition in the substance of the axon of the normal fibre, and therefore not demonstrable ordinarily with the heaxanitrite of cobalt and sodium, but that it is set free from the masked condition when the axon is injured.

It is, however, not to be supposed that the molecules of water do not penetrate the aggregates, for it is not, otherwise, possible to understand why the particles in organosols should exhibit such a degree of transparency in the ultramicroscopic apparatus, or why the addition of certain non-electrolytes should bring about precipitation of the particles. Indeed, one of the two phases possible in the relations of colloids to water necessarily postulates the presence of water molecules in the interior of the colloid particles.

The superficial portions of such particles must be regarded as exercising a considerable check on the process of diffusion from, or into, their interior, as the results of efforts made to obtain chloride-free globulins and albumins show. As already pointed out,† at least eight precipitations with ammonium sulphate are required to free the proteids of egg-albumen and serum from the associated chlorides, and this number suffices only if the precipitate after each precipitation is dissolved and kept in solution for a day at least before the next precipitate is brought about. When a much shorter time was allowed, for example, a couple of hours, the proteid of the twentieth precipitate still contained demonstrable quantities of chlorides.

These results seem to indicate that a part of the chlorides present is in such intimate association with the colloid particles that when these are brought into suspension (or "solution") in chloride-free water the chloride only slowly diffuse into the fluid surrounding or enveloping each particle. To account fully for such a result one may suppose that the chlorides, so difficult to remove, are in the fluid which obtains in the interior of the particles, the superficial layer of each of which is permeable (or impermeable) to such a degree as its extreme tenuity would permit.*

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* Zsigmondy, "Ueber Kolloidale Lösungen," 'Zeit. für Elektrochemie,' 1902, No. 32; Zsigmondy und Siedentopf, 'Annalen der Physik,' vol. 10, p. 1, 1903; also Gãtin-Gružewska and Biltz, 'Arch. für die ges. Physiol.,' vol. 105, 1904, p. 115.

† Macallum, 'Roy. Soc. Proc.,' B, vol. 76, p. 217, 1905.

Suspensions of such chloride-holding molecular aggregates in water† would manifest some degree of electrical conductivity, for if the suspended particles were numerous enough to form a more or less continuous series, the ionisation necessary for conductivity would occur, but the velocity of the ions, at least of those remaining in the interior of the particles, would be diminished. All this has a bearing on the question of nerve conductivity and on the nature of the nerve impulse itself.

The nerve axon consists in large part of colloids (proteids) in suspension, and in such a degree of concentration as to give a firm consistency to the axon.‡ In the particles of this suspension a part at least of the chloride or chlorides of nerve fibre, just as in ordinary egg-albumen, must be contained. Such a distribution of electrolytes would not permit the ions carrying the electrical charge to travel unimpeded and, in consequence, the change of potential transmitted would progress with greatly diminished velocity. This diminution would bring into line as parallel, if not very like, phenomena, the nerve impulse and the electrical current.

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* The same explanation may be advanced in the case of some inorganic colloids. In colloidal silica subjected to prolonged dialysis sodium compounds are present (Jordis and Kanter, 'Zeit. für anorg. Chemie,' vol. 34, p. 455, vol. 35, p. 16). In the case of colloidal ferric hydroxide "solution," Hantzsch ('Annalen der Chemie,' vol. 323, p. 1) found that silver nitrate did not precipitate all the chlorine present, and that the ferric hydroxide precipitate, obtained by the addition of salts, although very carefully washed, still contained chlorine, whose presence, however, could only be demonstrated after the washed precipitate was fused with soda. Spiro ('Beiträge zur Chem. Physiol. und Path.,' vol. 5, p. 276) found colloidal ferric hydroxide "solutions" even after months of dialysis to contain ionised as well as non-ionised chlorine, and, from quantitative analyses of the dialysed solutions, came to the conclusion that free hydrochloric acid is not present, and that colloidal ferric hydroxide is not a simple compound, but a substance of complicated constitution, or, much more probably, a mixture of compounds.

Is it not more reasonable to suppose that the peripheral layers of each colloid particle exercise a restraining influence on the transfer of the ions? That colloid material can manifest greatly diminished permeability to the ions, can be seen in an exaggerated degree in the case of the red blood corpuscle (ox and cat), in the cell-contents of which are electrolytes, but owing to impermeability of its membrane to ions it is a non-conductor (Bugarszky and Tangl, 'Centralbl. für Physiol.,' vol. 11, p. 297). And yet when such a corpuscle is placed in a hypotonic solution it yields to the latter not only its electrolytes, but also its hæmoglobin!

† It may be that the so-associated quantity of chlorides and, perhaps, of other inorganic compounds, equally difficult to remove, constitute the factor responsible for the very slight osmotic pressure and the very slight depression of the freezing point shown by presumably pure proteids.

‡ The hæmoglobin in the mammalian red corpuscle is 26 to 30 per cent., and the proteids of muscle approximate 20 per cent., but probably amount to more in the individual fibres. About 20 per cent. of solids would appear to be necessary to give the firm character to the axon which it possesses. It may be that the colloids and the water in the axon in their relations of solubility may occur in the two-phase system.

It may be freely admitted that one has not all the data necessary to establish certainty on this point, and that caution should be shown in drawing conclusions where so much is still unknown. The facts, however, stand out prominently. The occurrence of electrolytes, in the form of chloride or chlorides, in a concentrated degree, uniformly distributed in and along the course of the axon; the maintenance of this concentration through the impermeability of the medullary sheath and the neurilemma; the high conductivity of the axon; the occurrence of electrical phenomena on injury of the axon; these and other facts relating to the physical properties of nerve fibres justify one in proposing an explanation.

## EXPLANATION OF PLATES.

Figs. 1—9 are from silver nitrate preparations of nerve fibres, figs. 10—19 represent the action of mercurous nitrate on the same, while figs. 20—26 illustrate the action of the silver nitrate solution on capillary tubes filled with albumen or gelatine.

### PLATE 2.

FIG. 1.—*a* and *b* from the sciatic of the frog, *c* from the sciatic of the guinea-pig. $AgNO_3 \frac{N}{10}$ + 1·5 per cent. $HNO_3$, 24 hours. × 1000.

FIG. 2.—Sciatic fibres, frog. $AgNO_3 \frac{N}{10}$ + 1·5 per cent. $HNO_3$, 24 hours, thoroughly washed, then placed in 5 per cent. $CaCl_2$ solution: *a* for 2 hours, *b* for 8 hours. × 590.

FIG. 3.—Sciatic frog. $AgNO_3 \frac{N}{10}$ + 1·5 per cent. $HNO_3$, 24 hours, 5 per cent. $NaNO_3$ solution, 30 hours. *a* is the situation of a node of Ranvier. × 590.

FIG. 4.—From spinal cord of frog. $AgNO_3 \frac{N}{10}$ + 1·5 per cent. $HNO_3$. *a* × 3000, *b* × 670.

FIG. 5.—From spinal cord of frog. $AgNO_3 \frac{N}{10}$ + 1·5 per cent. $HNO_3$. × 2000.

FIG. 6.—*a* from spinal cord, *b* and *c* from sciatic nerve of frog. *a* and *b* illustrate the division of striæ. $AgNO_3 \frac{N}{10}$ + 10 per cent. $HNO_3$. × 3000.

FIG. 7.—From spinal cord of guinea-pig, illustrating both the diffuse and the striated (laminated) distribution of the chlorides of the axon. $AgNO_3 \frac{N}{10}$ + 1· per cent. $HNO_3$, 24 hours. × 1000.

FIG. 8.—From sciatic of rat, representing the distribution of the chlorides sometimes found after treatment with $AgNO_3 \frac{N}{10}$ + 10 per cent. $HNO_3$. × 590.

## PLATE 3.

FIG. 9.—From ganglionic cord of lobster, thoracic region ; *a* showing the diffuse chloride reaction in the axon, *b* the striate arrangement. $AgNO_3 \frac{N}{10}$ + 1·5 per cent. $HNO_3$ for 24 hours. × 1360.

FIG. 10.—*a*—*d*, from the sciatic of frog, illustrating the distribution of chlorides as revealed by the mercurous nitrate method. In *d* the clefts of Lanterman are seen as double funnels extending to the axon. Mercurous nitrate $\frac{S}{4}$ (S = saturation), 30 minutes, ammonium sulphide glycerine. × 590.

FIG. 11.—From the sciatic of frog, illustrating the diffuse and the laminated distribution of the chlorides, as revealed by the mercurous nitrate method. At *l* in *b* is seen a cleft of Lanterman, somewhat obscured by the precipitate, where apparently the reagent entered to produce the Frommann striation occurring on either side of it. $Hg_2(NO_3)_2 \frac{S}{4}$, 30 minutes, ammonium sulphide, glycerine. × 590.

FIG. 12.—From the sciatic of frog, illustrating the distribution of the chlorides, as revealed by different strengths of the reagent. *a*, $Hg_2(NO_3)_2 \frac{S}{4}$, 4 hours ; *b*, $Hg_2(NO_3)_2 \frac{S}{8}$, 4 hours, ammonium sulphide, glycerine. In *b* the chlorides are chiefly found on the surface of the axon. × 590.

FIGS. 13 and 14.—From the sciatic of frog. *a*, cleft of Lanterman, through which the reagent has penetrated to the axon and produced in the latter the Frommann striation. *n*, node of Ranvier, with the adjacent portion of the axon striated. $Hg_2(NO_3)_2 \frac{S}{4}$, 30 minutes, ammonium sulphide, glycerine. × 590.

FIG. 15.—Showing diffuse distribution of the chlorides in the axons of the sciatic of frog. *n*, node of Ranvier. $Hg_2(NO_3)_2 \frac{S}{4}$, 4 hours, ammonium sulphide, glycerine. × 590.

FIG. 16.—From the spinal cord of frog, illustrating a typical Frommann striation, as revealed by the mercurous nitrate method. $Hg_2(NO_3)_2 \frac{S}{4}$, 30 minutes, ammonium sulphide, glycerine. × 590.

FIG. 1.

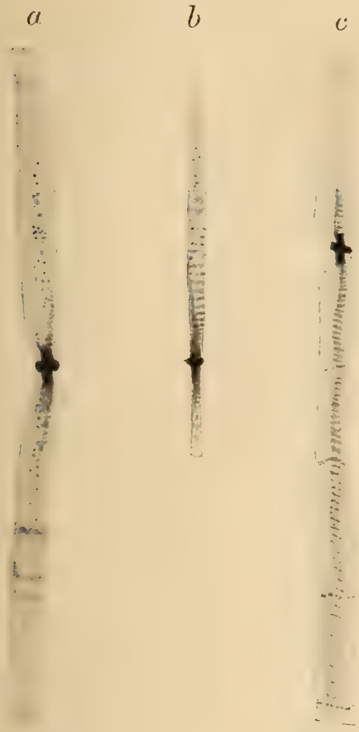


FIG. 2.

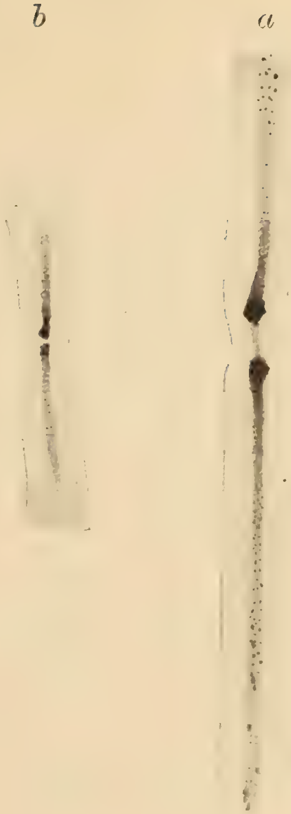


FIG. 3.

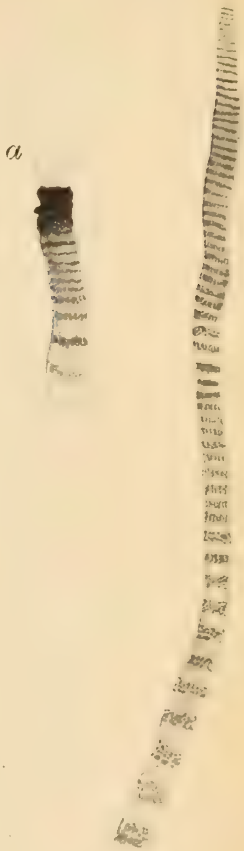


FIG. 4.

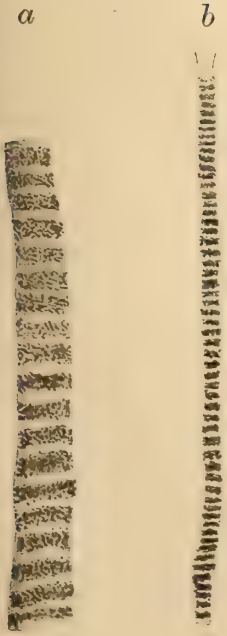


FIG. 5.

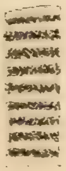


FIG. 6.

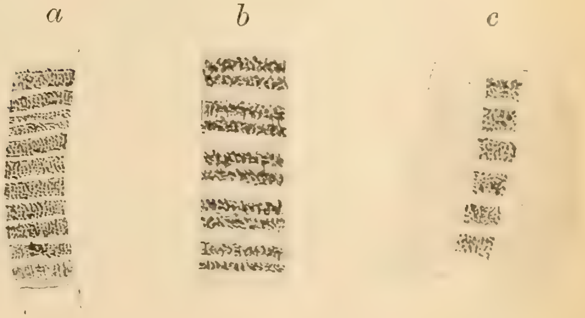


FIG. 7.

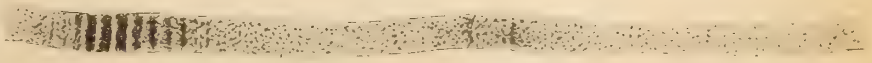


FIG. 8.

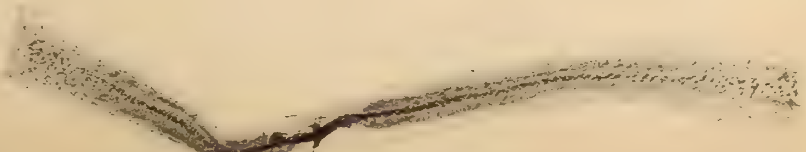


FIG. 9.

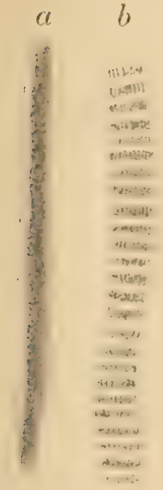


FIG. 10.

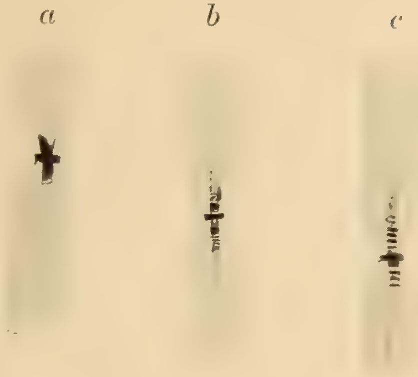


FIG. 12.

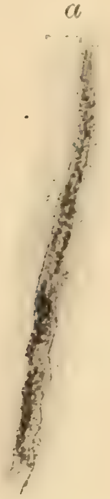


FIG. 13.

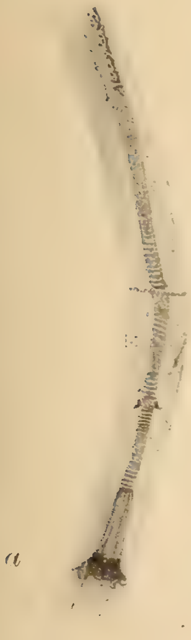


FIG. 10, *d*

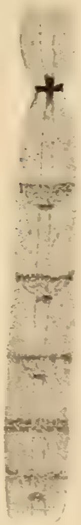


FIG. 11.

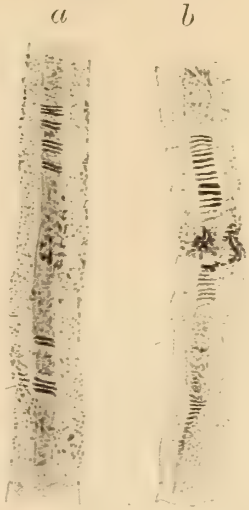


FIG. 12, *b*

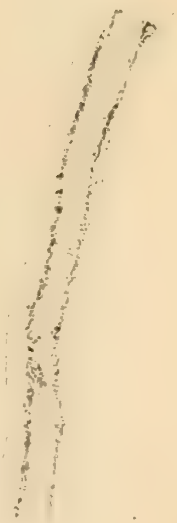


FIG. 16.

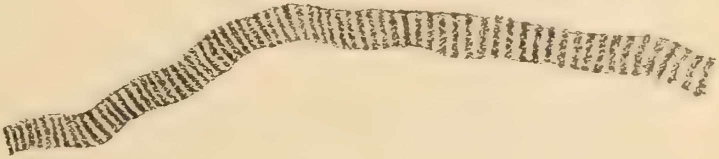


FIG. 14.

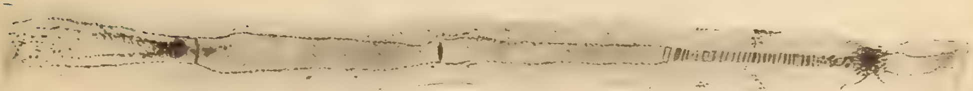


FIG. 15.

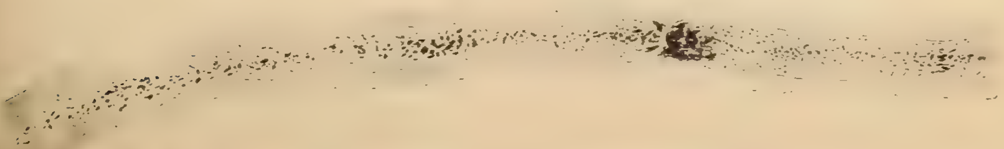


Fig. 17.

Fig. 18.

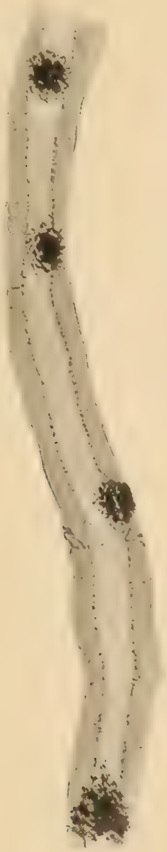


Fig. 19.

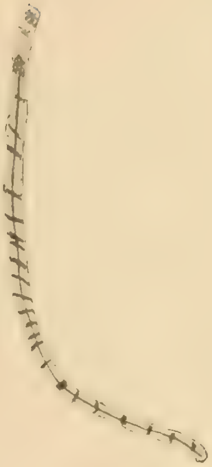


Fig. 20.

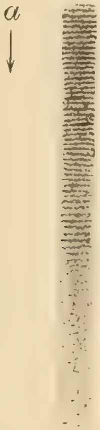


Fig. 21.

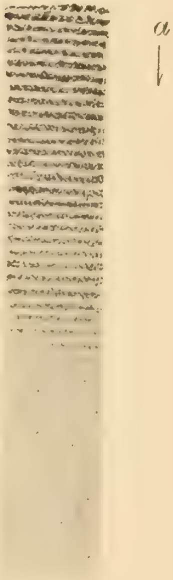


Fig. 22.

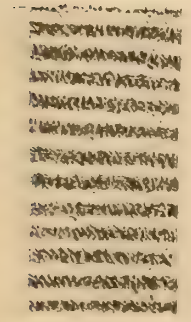


Fig. 23.

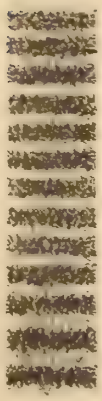


Fig. 24.

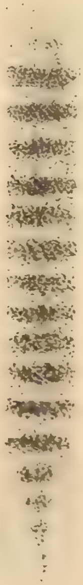


Fig. 25.

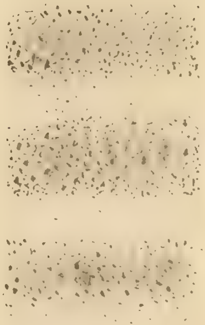
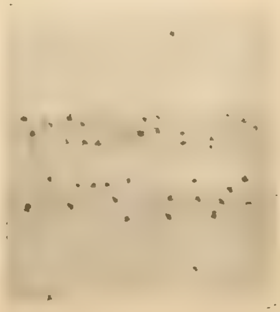


Fig. 26.



### PLATE 4.

FIG. 17.—From the sciatic of frog, showing the occurrence of chlorides in the imbrications of Lanterman. × 590.

FIG. 18.—From the sciatic of frog, illustrating a distribution of chlorides in the medullary sheath sometimes found. $Hg_2(NO_3)_2\,\frac{S}{4}$, 30 minutes, ammonium sulphide, glycerine. × 590.

FIG. 19.—From the sciatic of frog, illustrating the occurrence of chlorides in the imbrications of Lanterman and in the axon. $Hg_2(NO_3)_2\,\frac{S}{1}$, 2 hours, ammonium sulphide, glycerine. × 70.

FIG. 20.—Portion of end of capillary tube filled with albumen. $AgNO_3\,\frac{N}{10} + 1{\cdot}5$ per cent. $HNO_3$. The reagent has diffused in the direction of the arrow. × 105.

FIG. 21.—Portion of another capillary tube similarly treated. × 105.

FIG. 22.—Portion of a capillary tube filled with albumen and treated as in the case of fig. 20. × 590.

FIG. 23.—Portion of a capillary tube filled with albumen, to which sodium chloride was added to bring the strength of the salt in solution up to 1·73 per cent. Treated as in the case of fig. 20. × 70.

FIG. 24.—Portion of a capillary tube filled with gelatine containing 0·288 per cent. of sodium chloride, illustrating irregularities in the distribution of the silver precipitate. Treated as in the case of fig. 20. × 90.

FIGS. 25 and 26.—Portions of capillary tubes filled with commercial gelatine and treated as in the case of fig. 20, illustrating peculiarities in the precipitation of the silver chloride formed. Fig. 25 × 590, fig. 26 × 105.

# *On the Possibility of Determining the Presence or Absence of Tubercular Infection by the Examination of a Patient's Blood and Tissue Fluids.*

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(From the Pathological Laboratory, St Mary's Hospital, London, W.)

(Communicated by Sir John Burdon Sanderson, Bart., F.R.S. Received October 21,—Read November 23, 1905.)

In the present communication we propose (*a*) to set forth certain conclusions arrived at after the study of the tuberculo-opsonic power of the blood in a very considerable number of tubercular patients; (*b*) to show that we have in the measurement of the tuberculo-opsonic power of the blood and tissue fluids a method which may be exploited in the diagnosis of tubercular infection.

*Technique Employed.*—The technique employed by us in the measurement of the tuberculo-opsonic power of the blood was essentially that described by one of us in conjunction with Douglas.* In each case the white corpuscles required for the tests were derived from blood from the finger received into a solution of 0·5 per cent. citrate of soda in 0·85 NaCl, and rewashed after centrifugalisation in a considerable volume of 0·85 NaCl, and then again centrifugalised. Of the "blood-cream," obtained by skimming off the upper layer of the corpuscular sediment, one portion was in each case mixed in a capillary tube with one volume of serum and one volume of a suspension of tubercle bacilli which had been centrifugalised in such a manner as to free it from bacillary clumps. After incubation at 37° for 15 to 20 minutes films were made on slides prepared with emery paper.† These films were, after fixture in saturated corrosive sublimate, stained with boiling carbol-fuchsin, decolourised with 2 per cent. sulphuric acid, and counter-stained with methylene blue after washing in 1 in 1000 sodium carbonate. The standard of comparison employed was obtained by mixing in each case the same "blood cream" and tubercle suspension with "pooled normal serum."‡

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* 'Roy. Soc. Proc.,' vol. 72.

† Wright, 'Lancet,' July 9, 1904.

‡ While in this research pooled serum was employed, in order to provide against any chance variation of our bloods under the physical strain entailed by the work, it is to be noted that the observations of Urwick, conducted in this laboratory, and the more exten-

This "pooled serum" was obtained by mixing equal volumes of the sera of six to eight healthy students or laboratory workers. We have found that the opsonic power of such a "pooled serum" corresponds to the arithmetical mean of the opsonic indices of its component sera.

### *Classification of Tubercular Cases into Strictly Localised Cases, and Cases which are associated with Constitutional Disturbance.*

Cases of tubercular infection distribute themselves in a natural manner under two headings. Into one category would fall the patients who are the subjects of a strictly localised infection unaccompanied by anything in the nature of constitutional disturbance. Cases where the infection is limited to one or more lymphatic glands; further, most cases of lupus, most cases of tubercular abscess in the subcutaneous tissue, tubercular affections of the joints, and, lastly, many stationary or only slowly progressing cases of tubercular phthisis, fall into this category.

Into another category would fall patients who are suffering from more generalised tubercular infections associated with constitutional disturbance. This group consists in large part of cases of pyrexial pulmonary tuberculosis. With these may be classed certain other cases of extensive or widely disseminated tuberculosis.

### *Data with regard to the Tuberculo-opsonic Power in Cases of Strictly Localised Tuberculosis.*

The opsonic index is here low and uniformly low—in exceptional cases as low as one-sixth of the normal. Our findings in a series of cases of strictly localised tubercular infection are appended in tabular form below.

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sive series of investigations carried out by Bulloch at the London Hospital ('Medico-Chirurg. Soc. Proc.,' 1905), and Lawson and Stewart at the Banchory Sanatorium (*loc. cit.*), have conclusively shown—(*a*) That the tuberculo-opsonic power of the blood does not in health range below 0·9 or above 1·1; and (*b*) that the bloods of A. E. W., S. R. D., and others which have hitherto in this laboratory furnished a standard of comparison, are, from the point of view of their tuberculo-opsonic power, typically normal bloods.

Table I.—Showing the Tuberculo-opsonic Index in a Series of Cases of strictly localised Tuberculosis.

| Serial No. | Initials or, as the case may be, initial of patients. | Nature and seat of the infection. | Tuberculo-opsonic index. |
|---|---|---|---|
| 1 | J. R. | Tubercle of testis | 0·65 |
| 2 | A. | Caries, lower end of femur | 0·7 |
| 3 | C. S. | Tubercular ulceration, dorsum of hand | 0·86 |
| 4 | A. B. | „ iritis. | 0·51 |
| 5 | B. C. | „ glands | 0·4 |
| 6 | E. M. | „ ulceration of legs. 13 years' duration | 0·17 |
| 7 | H. W. | „ glands (neck) | 0·82 |
| 8 | D. W. | „ „ „ | 0·64 |
| 9 | S. | „ ulceration of legs | 0·49 |
| 10 | L. B. | „ glands (abdominal) | 0·13 |
| 11 | B. | „ kidney | 0·75 |
| 12 | M. H. | „ cystitis | 0·85 |
| 13 | D. B. | „ glands. Extirpated and re-appeared | 0. 85 |
| 14 | M. O. E. | „ glands | 0·6 |
| 15 | W. | Psoas abscess | 0·75 |
| 16 | C. H. | „ „ | 0·65 |
| 17 | W. | Tubercular glands (neck). 18 months' duration | 0·47 |
| 18 | C. | „ wrist | 0·85 |
| 19 | U. R. | „ glands (neck) | 0·7 |
| 20 | A. H. | „ glands. Extirpated and re-appeared | 0·54 |
| 21 | P. | „ peritonitis | 0·6 |
| 22 | R. | „ abscesses and glands | 0·6 |
| 23 | E. | Lupus | 0·6 |
| 24 | R. | Tubercle of testes and bladder | 0·72 |
| 25 | P. | Tubercular peritonitis | 0·7 |
| 26 | H. | „ caries of fibula | 0·6 |
| 27 | C. | Tubercle of kidney | 0·88 |
| 28 | W. | Tubercular disease of knee | 0·6 |
| 29 | T. | „ glands (neck) recurrence | 0·66 |
| 30 | S. | „ ulcer of foot | 0·49 |
| 31 | C. | „ disease of knee | 0·75 |

### *Data with regard to the Tuberculo-opsonic Power of the Blood in Cases of Tuberculosis associated with Constitutional Disturbance.*

In the cases here in question the opsonic index of the blood is continually varying. The range of its fluctuation is from considerably under the normal to twice or more the normal height.

Striking examples of the variation of the opsonic index in connection with acute tubercular phthisis are furnished in the paper of our fellow-worker, R. H. Urwick, already referred to.*

The following are instances of similar variation occurring in the subjects of other forms of tubercular infection:—

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* 'British Medical Journal,' July 22, 1905.

Example 1.—Child with Tubercular Caries of the Fibula, associated with Constitutional Disturbance.

| Dates of blood examinations. | Tuberculo-opsonic index. |
|---|---|
| 11.9.05 | 1·45 |
| 14.9.05 | 1·71 |
| 19.9.05 | 1·3 |
| 28.9.05 | 0·98 |
| 30.9.05 | Operation, fibula scraped. |
| 2.10.05 | 1·73* |
| 3.10.05 | 1·13 |
| 10.10.05 | 1·3 |

Example 2.—Adult Patient with Tubercular Caries of the Spine and Constitutional Disturbance.

| Date of blood examination. | Tuberculo-opsonic index. | Remarks. |
|---|---|---|
| 19.5.05......... | 0·65 | Temperature disturbance and pain associated with development of abscess. |
| 20.5.05......... | 1·4 | |
| 22.5.05......... | 1·3 | |
| 23.5.05......... | 1·0 | Temperature returns to normal in association with spontaneous discharge of abscess. |
| 24.5.05......... | 0·8 | |

Example 3.—Adult Patient with extensive Psoas Abscess and Generalisation of Tubercle. Case has since terminated fatally.

| Date of blood examinations. | Tuberculo-opsonic index. |
|---|---|
| 8.2.05 | 2 |
| 9.2.05 | 2·4 |
| 11.2.05 | 0·6 |

*Suggested Interpretation of the Different Findings in these two Categories of Cases.*

The explanation of the difference in the condition of the blood in these two contrasted categories of cases is probably the following: The condition of low opsonic power which is associated with strictly localised tuberculosis is almost certainly a condition which has preceded and has furnished the opportunity for infection. The fact that the opsonic index continues persistently low after infection has supervened, while it can invariably be raised by appropriate inoculation,* indicates that the machinery of immunisation with which the organism is furnished is not, under the conditions which obtain in strictly localised tubercular infections, spontaneously called into play.

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*A rise in the opsonic power similar to this here registered has been repeatedly observed by us in connection with the stirring up by surgical interference of tubercular foci.

The constant fluctuation in the opsonic power of the blood in cases of active pulmonary tuberculosis and other active forms of tubercular infection furnishes—as we can hardly doubt—evidence of a periodic conveyance of tubercular elements into the blood; and of a response to such stimulation on the part of the machinery for immunisation. The low opsonic indices registered in connection with active tuberculosis would in other words be "negative phases" such as supervene—as one of us has shown—upon the inoculation of all vaccines; the high opsonic indices would be "positive phases," such as normally succeed upon the negative phases just mentioned; and the normal opsonic indices would correspond to periods of transition between negative and positive phases, or, as the case may be, to periods in which the blood is returning after a positive phase to the condition *quo ante.*

The life of a patient with any really active form of tuberculosis would in conformity with this view be a life of alternating negative and positive phases: the favourable or unfavourable event of the infection being in each case determined by the adjustment or want of adjustment of the auto-inoculations (with respect to dosage and interspacing) with the particular patient's capacity for immunising response.

Having now to a certain extent cleared the ground, we may pass on to consider the question of the diagnosis of tubercular infection by means of the measurement of the opsonic power of the blood.

### *Exploitation of the data Summarised above as an aid in the Diagnosis of Tubercular Infection.*

Consideration will make clear that the data obtained by the measurement of the opsonic power in cases of doubtful diagnosis may, when adjudicated upon in the light of the data obtained in connection with undoubted cases of tuberculosis as given above, furnish material for admitting or rejecting the diagnosis of tubercular infection. We may formulate in connection with this matter the following propositions:—

(1) Conclusions which can be arrived at when we have at disposal the results of a series of measurements.

(a) *Where a series of measurements of the opsonic power of the blood reveals a*

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* Exactly the same statements hold true with regard to the staphylo-opsonic power in localised staphylococcus infections (furunculosis, sycosis, etc.).

*persistently low opsonic power with respect to the tubercle bacillus, it may be inferred, in the case where there is evidence of a localised bacterial infection which suggests tuberculosis, that the infection in question is tubercular in character.*

(b) *Where repeated examination reveals a persistently normal opsonic power with respect to the tubercle bacillus, the diagnosis of tubercle may with probability be excluded.*

Illustrative case: A. B.—Case diagnosed as tubercular cystitis on the evidence of pus in the urine, of the cystoscopic appearances and general disturbance of health. The measurement of the tuberculo-opsonic power of the blood yielded the following results:—

| Date of blood examination. | Tuberculo-opsonic index. |
|---|---|
| 2.3.05 | 0·98 |
| 14.4.05 | 0·99 |
| 28.4.05 | 1 |
| 18.5.05 | 1 |
| 19.5.05 | 1·1 |
| 2.10.05 | 0·97 |

The inference that the cystitis and disturbance of health was not of tubercular origin was confirmed (*a*) by the fact that an extensive series of bacteriological examinations prolonged over many months revealed in every case the presence of proteus in large numbers, while the tubercle bacillus was never found, even when examined for by the inoscopic method of Jousset; (*b*) by the fact that the patient's blood possessed, anterior to treatment with regard to the proteus, an agglutinating power which was three times higher than the normal; and (*c*) by the fact that very striking amelioration of the cystitis, and a complete return to health has been obtained as the result of the inoculation of a proteus vaccine.

(c) *Where there is revealed by a series of blood examinations a constantly fluctuating opsonic index the presence of active tuberculosis may be inferred.*

C. D.—A case of severe chronic urticaria of unknown ætiology. The measurement of the tuberculo-opsonic power of the patient's blood yielded the following results:—

| Date of blood examination. | Tuberculo-opsonic index. |
|---|---|
| 20.5.05 | 1·3 |
| 26.5.05 | 1·3 |
| 16.6.05 | 0·86 |
| 20.6.05 | 1·27 |

The inference drawn from these data that the patient was suffering from some active form of tuberculosis was confirmed (*a*) by the discovery by an independent observer of a lesion in the apex of one lung; (*b*) by the subsequent development of an abscess of an obviously tubercular character; and (*c*) by the marked improvement in health which has followed upon inoculation with tubercle vaccine.

(2) Conclusions which may be arrived at where we have at disposal the result of an isolated blood examination.

(a) *Where an isolated blood examination reveals that the tuberculo-opsonic power of the blood is low, we may—according as we have evidence of a localised bacterial infection or of constitutional disturbance—infer with probability that we are dealing with tuberculosis—in the former case with a localised tubercular infection, in the latter with an active systemic infection.*

(b) *Where an isolated blood examination reveals that the tuberculo-opsonic power of the blood is high, we may infer that we have to deal with a systemic tuberculous infection which is active, or has recently been active.*

(c) *Where the tuberculo-opsonic power is found normal, or nearly normal, while there are symptoms which suggest tuberculosis, we are not warranted, apart from the further test described below, in arriving at a positive or a negative diagnosis.*

### *Discrimination of Tubercular Blood from Normal Blood by the aid of the Phagocytic Test Conducted with Serum which has been subjected to a Temperature of* 60° *C.*

The further criterion to which reference was made in the preceding paragraph is the following:—

*When a serum is found to retain in any considerable measure, after it has been heated to* 60° *for* 10 *minutes, its power of inciting phagocytosis we may conclude that "incitor elements"** *have been elaborated in the organism either in response to auto-inoculations occurring spontaneously in the course of tubercular infection, or, as the case may be, under the artificial stimulus supplied by the inoculation of tubercle vaccine.*

A typical selection from the very extensive body of observations which furnishes the basis of the above statement is presented in Tables II and III.

It will be seen from these tables that in practically every case where a reaction to tubercular infection may be assumed to have taken place,

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* The term "incitor elements" (Latin, *incito*, I urge forward, I hasten, I bring into rapid movement) is here employed in lieu of a more specific term, in order not to prejudge the mode of action of the element in the heated serum which promotes phagocytosis. The nature of the incitor element is considered in the next following communication.

Table II.—Showing that the Normal Serum, after it has been exposed to a Temperature of 60° C. for 10 minutes, no longer incites Phagocytosis.*

| Serial number of the observation. | Derivation of the serum. | Unheated serum. | | Heated serum. | |
|---|---|---|---|---|---|
| | | Phagocytic count. (Number of bacteria ingested divided by number of leucocytes examined.) | Tuberculo-opsonic index. | Phagocytic count. (Number of bacteria ingested divided by number of leucocytes examined.) | Tuberculo-opsonic index. |
| 1 | Healthy man ......... | (104/40) = 2·6 | Taken as 1 | (13/40) = 0·32 | 0·125 |
| 2 | " ......... | (96/40) = 2·4 | " 1 | (8/40) = 0·2 | 0·08 |
| 3 | Pooled serum of six healthy men | (247/36) = 6·8 | " 1 | (30/50) = 0·6 | 0·09 |
| 4 | Healthy boy............ | (250/39) = 6·4 | " 1 | (15/40) = 0·4 | 0·06 |
| 5 | " ............ | (214/30) = 7·0 | " 1 | (19/40) = 0·47 | 0·06 |
| 6 | Pooled serum of eight normal men | (60/50) = 1·2 | " 1 | (2/20) = 0·1 | 0·08 |
| 7 | Healthy man ......... | (55/40) = 1·4 | " 1 | (0/40) = 0·0 | 0·00 |
| 8 | Pooled serum of six healthy men | (132/30) = 4·4 | " 1 | (3/30) = 0·1 | 0·1 |

evidence of that reaction can be obtained by conducting the phagocytic test with serum which has been heated to 60° C. for 10 minutes.

The observations numbered 15 and 16 respectively have, it may be noted, been introduced into the table with the special design of showing the very simple nature of the investigation which is required for the diagnosis of tubercle in the case where that infection has called forth a reaction of immunisation.

The following observations, which we owe to our fellow-worker Dr. G. W. Ross, bring out in an instructive manner the trustworthiness of the phagocytic test with heated serum as applied in this its simplest form :—

### *Case* 1.—*Girl, æt. six years, Tentatively Diagnosed Pulmonary Phthisis.*

Phagocytosis obtained with the serum, heated for 10 minutes to 60° C. and employed in a phagocytic mixture containing over 1 per cent. NaCl.

The verdict of tubercular infection of the lung which was based on this was confirmed on *post-mortem* examination.

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* In order to avoid the fallacies associated with spontaneous phagocytosis (*vide* the next following communication) the observations which are recorded in this and in the subsequent table were in each case made by mixing the volume of the serum with one volume of corpuscles, washed in 0·85-per-cent. NaCl solution and one volume of tubercle bacilli suspended in a 1·5-per-cent. NaCl solution. In this manner a salt content of over 1-per-cent. NaCl was achieved in the phagocytic mixture.

Table III.—Showing that an element which incites Phagocytosis is contained in the heated serum of patients who are the subjects of an active systemic tubercular infection, or who have been subjected to inoculations of a tubercle vaccine. The sera, like those which are in question in Table II, were in each case heated to 60° C. for 10 minutes.

| Serial number of observation. | Nature of infection. | Unheated serum. | | Heated serum. | |
|---|---|---|---|---|---|
| | | Phagocytic count. (Number of bacteria ingested divided by number of leucocytes examined.) | Tuberculo-opsonic index (determined by comparison of phagocytic count with that obtained with pooled blood of healthy men). | Phagocytic count. (Number of bacteria ingested divided by number of leucocytes examined.) | Tuberculo-opsonic index (determined by comparison of phagocytic count with that obtained with unheated pooled blood of normal men). |
| 1 | Tubercular caries of hip | — | 1·5 | — | 0·4 |
| 2 | Tubercular phthisis ... | (125/20) = 6·2 | 1·4 | (113/30) = 3·7 | 0·8 |
| 3 | „ ... | (152/30) = 5·0 | 1·2 | (96/30) = 3·2 | 0·72 |
| 4 | „ ... | (98/30) = 3·2 | 1·0 | (20/65) = 0·3 | 0·1 |
| 5 | Tubercular peritonitis | (144/30) = 4·8 | 1·4 | (103/30) = 3·4 | 1·0 |
| 6 | „ ... | (142/30) = 4·7 | 1·4 | (16/50) = 0·3 | 0·09 |
| 7 | Phthisis and tubercular glands | (113/40) = 2·8 | 1·1 | (79/50) = 1·6 | 0·6 |
| 8 | Tubercular caries of hip | (110/30) = 3·6 | 1·0 | (85/30) = 2·8 | 0·8 |
| 9 | Tubercular abscess of kidney | — | 1·7 | (26/30) = 0·8 | 0·4 |
| 10 | Lupus under treatment by inoculation of tubercle vaccine | (34/10) = 3·4 | 0·7 | (49/30) = 1·6 | 0·33 |
| 11 | Tubercular ulcer of leg under treatment by inoculation of tubercle vaccine | (249/40) = 6·2 | 1·2 | (149/40) = 3·7 | 0·7 |
| 12 | Tubercle of kidney under treatment by inoculation of tubercle vaccine | (68/40) = 1·7 | 1·5 | (77/40) = 1·9 | 1·7 |
| 13 | Tubercular glands and abscess under treatment by inoculation of tubercle vaccine | (59/40) = 1·5 | 1·4 | (36/40) = 0·9 | 0·8 |
| 14 | Tubercular cystitis under treatment by inoculation of tubercle vaccine | (97/50) = 2·0 | — | (43/30) = 1·4 | — |
| 15 | Phthisis ................. | — | — | (26/30) = 0·8 | — |
| 16 | „ .................. | — | — | (9/5) = 1·8 | — |

### *Case 2.—Man, æt. 41, Tentative Diagnosis, Pleurisy due to Malignant Disease, or Tubercular Pleurisy.*

No phagocytosis obtained with the serum, heated for 10 minutes to 60° C. and employed in a phagocytic mixture containing over 1 per cent. NaCl.

The verdict of pleurisy due to malignant disease, which was based on this, was confirmed on *post-mortem* examination.

### *Case 3.—Case Tentatively Diagnosed Miliary Tuberculosis or Malignant Endocarditis.*

No phagocytosis obtained with the serum, heated for 10 minutes to 60° C. and employed in a phagocytic mixture containing over 1 per cent. NaCl.

The verdict of malignant endocarditis which was based on this was confirmed on *post-mortem* examination.

*Observation 4.—Case Diagnosed Miliary Tuberculosis.*

No phagocytosis obtained with the serum, heated for 10 minutes to 60° C. and employed in a phagocytic mixture containing over 1 per cent. of NaCl.

The *post-mortem* examination revealed a complete absence of tubercular lesions and a healing typhoid ulcer* in the ileum.

### *On two other Methods by which a Diagnosis of Tubercular Infection can be arrived at or Excluded.*

In addition to the methods which have been already considered, there are two further methods which can be exploited in connection with the diagnosis of tubercular infection. The first of these is applicable where we desire to supplement the often ambiguous data furnished by the clinical symptoms in the case of inoculations of tuberculin undertaken for diagnostic purposes. The second is applicable where we can obtain, in addition to the patient's blood, also lymph, or, as the case may be, pus from the seat of infection.

### *Diagnosis of Tubercular Infection by the Aid of Measurements of the Opsonic Power carried out in Connection with the Inoculation of Tuberculin for Diagnostic Purposes.*

Already, three years ago,† in connection with a paper on staphylococcus inoculations as applied to the treatment of acne, furunculosis, and sycosis, attention was directed by one of us to the close analogy between the tubercular reaction of Koch and the local inflammation and general constitutional disturbance which may supervene when a patient whose tissues are extensively invaded by the staphylococcus is inoculated with the corresponding vaccine in such a manner as to develop a pronounced negative phase.

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* A negative Durham-Gruber reaction had been obtained in this case.

† 'Lancet,' March 29, 1902.

The association of a negative phase with a reaction similar to that conveniently spoken of as the *tuberculin reaction,* suggested to us the propriety of enquiring whether the true tuberculin reaction, as seen after the injection of Koch's old tuberculin into a tubercular patient, was also associated with a negative phase.

The opportunities for investigating the question which have presented themselves have not yet been sufficiently numerous to allow of our formulating a final answer to this question. The observations which are set forth below seem to us to suggest that the development of a negative phase, with a dose of tuberculin smaller than that which would produce this result in a healthy patient, may prove to be an index of tubercular infection. Such a conclusion would be in harmony with our experience in connection with the therapeutic inoculation of tubercle vaccine (new tuberculin). We find in this connection that the negative phase supervenes upon very much smaller doses and persists much longer in the case where the patient is the subject of extensive infection than in the contrary case.

*Observation* 1.—Case diagnosed, Tubercular choroiditis.

| Date. | Tuberculo-opsonic index. | Clinical data. |
|---|---|---|
| 26.4.05............ | 0·9 | |
| 5 milligrammes old tuberculin inoculated. | | |
| 29.4.05............ | 0·29 | Some constitutional reaction, *t.* 100° F. |
| 28.4.05............ | 0·95 | |

*Observation* 2.—Case diagnosed, Lupus erythematosus.

| Date. | Tuberculo-opsonic index. | Clinical data. |
|---|---|---|
| 12.1.05............ | 0·73 | |
| Inoculation of 1 milligramme old tuberculin. | | |
| 13.1.05............ | 0·85 | No rise of temperature or constitutional or local reaction. |
| 17.1.05............ | 1·6 | |
| 26.1.05............ | 0·5 | |

*Observation* 3.—Case diagnosed, Lupus erythematosus.

| Date. | Tuberculo-opsonic index. | Clinical data. |
|---|---|---|
| 10.4.05............ | 0·66 | |
| Inoculation of 5 milligrammes of old tuberculin. | | |
| 11.4.05............ | 0·7 | Quite insignificant constitutional disturbance. |
| 12.4.05............ | 1·2 | |
| 14.4.05............ | 0·85 | |

*Observation* 4.—Case diagnosed as Lupus vulgaris.

| Date. | Tuberculo-opsonic index. | Clinical data. |
|---|---|---|
| 10.4.05............ | 0·55 | |
| Inoculation of 5 milligrammes of old tuberculin. | | |
| 11.4.05............ | 1·1 | Quite insignificant constitutional reaction. |
| 12.4.05............ | 1·0 | |
| 14.4.05............ | 1·0 | |

*Observation* 5.—Lupus, patient had been treated for many months by therapeutic inoculations of tubercle vaccine.

| Date. | Tuberculo-opsonic index. | Clinical data. |
|---|---|---|
| 24.1.05............ | 1·4 | |
| Inoculation of 30 milligrammes of tuberculin. | | |
| 25.1.05............ | 0·34 | Severe constitutional and local reaction, *t.* of 103° F. |
| 26.1.05............ | 2·1 | |
| 27.1.05............ | 1·7 | |

### *Diagnosis of Tubercular Infection by the Comparison of the Opsonic Power of the Patient's Blood with the Tuberculo-opsonic Power of the Fluids Derived from the Focus of Infection.*

Attention has already been drawn by one of us, both in a research undertaken in conjunction with Lamb* and in a research undertaken in conjunction with Douglas,† to the fact that we have in the actual focus of infection a lowered "bacteriotropic pressure" which accounts for the cultivation of the pathogenetic microbe in the interior of an organism which has at disposal in the circulating blood a considerable reserve of anti-bacterial substances. We propose here in conclusion to furnish further illustration of the general law as enunciated above, culling our examples not alone from the observations we have made in connection with tubercular infection, but also from observations made in connection with other bacterial infections.

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* 'Lancet,' December 23, 1899.

† 'Roy. Soc. Proc.,' vol. 74, p. 157.

*Observation* 1.—*Case of abscess in the neighbourhood of the appendix.* Blood from the patient's finger and pus obtained from the abscess at the operation were examined, with a view to determining the nature of the infection.

| | Phagocytic counts. | |
|---|---|---|
| | With a suspension of tubercle bacilli. | With a suspension of staphylococci. |
| Serum ........................ | 2·3 | 4·5 |
| Fluid obtained from the pus by centrifugalisation | 0·1 | 1·9 |

The fact that the tuberculo-opsonic power of the patient's blood was here 23 times as great as that of the fluid obtained from the pus was taken as evidence that tuberculo-opsonic substances had been used up in the pus and that the patient was suffering from a tubercular infection. It was inferred on similar grounds that he was also infected by staphylococcus.

*Observation* 2.—*Case of osteo-myelitis of the femur.* Blood from the patient's finger and pus obtained from the abscess at the operation were examined, with a view to determining the nature of the infection.

| | Tuberculo-opsonic index. | Staphylo-opsonic index. |
|---|---|---|
| Serum ........................ | 1·0 | 2·5 |
| Fluid obtained from the pus by centrifugalisation | 1·1 | 0·9 |

The fact that the opsonic index of the patient's circulating blood was here normal to tubercle, while it was two and a-half times greater than normal with respect to the staphylococcus, was taken as evidence that the patient was not infected with tubercle, and that he was infected by staphylococcus, and had responded to that infection by a production of immunising substances.

The fact that the tuberculo-opsonic index of the fluids obtained from the pus was the same as that of the blood, while the staphylo-opsonic power was only two-fifths of that of the circulating blood, was taken as of confirmatory evidence of the conclusion already arrived at. The fact that a copious culture of staphylococcus aureus was obtained from the pus, planted out with aseptic precautions at the operation, further confirmed the diagnosis.

*Observation* 3.—*Case of psoas abscess.* Blood from the patient's finger and pus from the abscess were examined.

| | Phagocytic counts. | |
|---|---|---|
| | With a suspension of tubercle bacilli. | With a suspension of staphylococci. |
| Serum ........................ | 2·4 | 5·0 |
| Fluid obtained from the pus by centrifugalisation | 1·23 | 1·2 |

The fact that the fluid obtained from the pus was impoverished in both tuberculo- and staphylo-opsonic substances as compared with the blood was taken as evidence of a combined infection by tubercle bacilli and staphylococci. This inference was confirmed by the fact that the opsonic power of the blood with respect to both the micro-organisms here in question was undergoing perpetual fluctuations.* The inference so far as it related to the staphylococcus was further confirmed by the fact that cultures of the micro-organism were obtained from the pus.

*Observation* 4.—*Case of ascites with grave constitutional disturbance in a man of* 30. Blood from the finger and ascitic fluid were examined on two occasions.

First Occasion.

| | Tuberculo-opsonic index. |
|---|---|
| Serum........................ | 1·05 |
| Ascitic fluid ............ | 0·99 |

We reported upon this that the patient was not suffering from tubercular peritonitis.

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* For the variations registered in connection with the tuberculo-opsonic power, *vide supra*, p. 196 of this paper, where Example 3 refers to the patient here in question.

The clinical symptoms, the age of the patient, and the appearances as seen at the operation appearing in contradiction with this verdict, and the ascites having reappeared, a second operation was performed, and a further sample of ascitic fluid was obtained for examination. At the same time the clinical appearances were again observed, with the result that there was now some wavering as to whether the original diagnosis could be upheld. The result of the phagocytic examination of the ascitic fluid, and of a sample of blood from the fingers were now as under:—

| | Tuberculo-opsonic index. |
|---|---|
| Serum | 1 |
| Ascitic fluid | 1 |

In view of this result the verdict previously given was sustained.

A *post-mortem* examination, which followed in the course of a few weeks, again threw doubt on the verdict, the naked-eye appearances being entirely consistent with the theory of miliary tuberculosis affecting the peritoneum and serous covering of the intestines. Microscopic examination of the sections made through the miliary nodules revealed, however, a typical picture of miliary carcinoma. No primary carcinomatous focus had been discovered, though it was sought for on *post-mortem* examination.

*Observation* 5.—*Case of pleural effusion.* Blood from the finger and fluid obtained by paracentesis of chest were examined:—

| | Tuberculo-opsonic index. |
|---|---|
| Serum | 0·92 |
| Pleural fluid | 1·0 |

This was taken as evidence of the absence of tubercular infection.

*Observation* 6.—*Case diagnosed as peritoneal tubercular peritonitis complicated with pleurisy.* Blood from the finger was examined on two occasions. On the second occasion, which was 48 hours after the first examination, peritoneal and pleural fluid were also examined.

The results obtained by the phagocytic examination undertaken on this second occasion were as follows:—

| | Tuberculo-opsonic index. |
|---|---|
| Serum | 0·7 |
| Peritoneal fluid | 0·28 |
| Pleural fluid | 1 |

The results of the comparison of the peritoneal fluid with the serum obtained from the blood withdrawn from the finger were taken as evidence of tubercular infection of the peritoneum. Confirmatory evidence of tubercular infection was furnished further by the low tuberculo-opsonic power of the blood, and by the observed fluctuation in this index. When it was measured two days previously, this index had worked out as 1·4.

The fact that the opsonic power of the pleural fluid worked out as higher than the opsonic power of the serum was taken as evidence that the pleural effusion had occurred at a period when the opsonic power of the blood was 1 or above 1.

The diagnosis of tubercular infection of the peritoneum and pleura (and underlying lung) was confirmed at the *post-mortem* examination.

*Observation* 7.—*Case of long-continued suppuration of the antrum* associated with the presence in the pus of the pneumococcus and the *Bacillus fusiformis* and *Spirillum buccæ* of Vincent. The patient had been treated by therapeutic inoculations of a pneumococcus vaccine. The patient's serum and the antral pus were examined with a view to determining whether the pneumococcus played any active part in connection with the continuance of the suppuration :—

| | Pneumo-opsonic index. |
|---|---|
| Serum | 4·3 |
| Fluid obtained from pus by centrifugalisation | 0·3 |

The results were taken as evidence (*a*) that the pneumococcus played an active *rôle* in connection with the suppuration, and (*b*) that the protective substances which had been generated in the blood under the influence of inoculation did not come satisfactorily into application upon the micro-organisms in the antrum.

*Observation* 8.—*Case of whitlow associated with the formation of a blister under the nail.* Serum derived from blood from a sound finger and blister fluid were examined.

| | Staphylo-opsonic index. |
|---|---|
| Serum | 0·8 |
| Blister fluid | 0·3 |

The blister fluid yielded a pure culture of staphylococcus.

*Observation* 9.—*Rabbit in the early stages of anthrax infection.*—Blood obtained from the ear and lymph from the seat of inoculation were examined.

| | Anthraco-opsonic index.* |
|---|---|
| Serum | 1·7 |
| Lymph | 0·62 |

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* Tested with a suspension of anthrax spores and compared with the serum of a normal rabbit tested in the same manner.

It may be noted that all the difficulties and inaccuracies which are associated with the employment of ordinary anthrax cultures in phagocytic experiments can be satisfactorily evaded by the employment of suspensions of anthrax spores. These, when stained with carbol fuchsin and decolourised by 0·25 per cent. sulphuric acid, represent absolutely ideal elements for enumeration.

## APPENDIX.

*A further Series of Observations showing that Phagocytosis is obtained with the Heated Serum of Patients who are the subjects of a Systemic as distinguished from a strictly Localised Tubercular Infection, or who, being the subjects of a strictly Localised Tubercular Infection, have been subjected to Inoculations with Tubercle Vaccine. The serum was in each case heated to* 60° *C. for* 10 *minutes.*

Table supplementary to Table II.—Showing that the Normal Serum, after it has been exposed to a Temperature of 60° C. for 10 minutes, no longer incites Phagocytosis.

| Serial number of observation. | Nature of infection. | Unheated serum. | | Heated serum. | |
|---|---|---|---|---|---|
| | | Phagocytic count. (Number of bacteria ingested divided by number of leucocytes examined.) | Opsonic index (determined by comparison of phagocytic count with that obtained with pooled blood of healthy men). | Phagocytic count. (Number of bacteria ingested divided by number of leucocytes examined.) | Opsonic index (determined by comparison of phagocytic count with that obtained with pooled unheated blood of normal men). |
| 1 | Fibroid phthisis, tubercle bacilli in sputum | (100/30) = 3·3 | 1·0 | (142/37) = 4·0 | 1·2 |
| 2 | Early phthisis, tubercle bacilli in sputum | (132/30) = 4·4 | 1·3 | (122/47) = 2·6 | 0·77 |
| 3 | Acute phthisis, tubercle bacilli in sputum | (130/30) = 4·3 | 1·3 | (96/40) = 2·4 | 0·74 |
| 4 | Acute phthisis ……… | (127/40) = 3·2 | 1·0 | (45/34) = 1·3 | 0·4 |
| 5 | Fibroid phthisis (?) … | (182/30) = 6·0 | 1·8 | (51/43) = 1·2 | 0·3 |
| 6 | Phthisis, tubercle bacilli in sputum | (117/30) = 3·9 | 1·1 | (65/30) = 2·2 | 0·62 |
| 7 | Mitral stenosis ……… | (106/30) = 3·5 | 1·0 | (19/31) = 0·6 | 0·17 |
| 8 | Early phthisis ……… | (161/30) = 5·4 | 1·6 | (54/27) = 2·0 | 0·6 |
| 9 | Phthisis ……………… | (257/40) = 6·4 | 1·3 | (51/40) = 1·3 | 0·27 |
| 10 | Lupus under treatment by inoculation of tubercle vaccine | (131/36) = 3·3 | 1·6 | (74/40) = 1·8 | 0·8 |
| 11 | Lupus under treatment by inoculation of tubercle vaccine | (73/30) = 2·4 | 1·2 | (31/30) = 1·0 | 0·5 |
| 12 | Tubercular ulcer of leg under treatment by inoculation of tubercle vaccine | (63/30) = 2·1 | 1·2 | (60/30) = 2·0 | 1·1 |

The first eight of the observations here in question were made upon bloods collected for us in the Victoria Park Hospital by our fellow worker, Dr. G. W. Ross. The clinical diagnosis which had been arrived at was not made known to us till afterwards, when the particulars set forth in Column 2 were filled in by Dr. Ross.

In contrast with the observations incorporated in Table II in the body of the paper these observations were conducted in phagocytic mixtures containing 0·85 per cent. instead of 1·1 per cent. of NaCl. It is shown in the next following communication that spontaneous phagocytosis is absolutely abolished only in the case when the salt content of the phagocytic mixture exceeds 1 per cent.

The source of fallacy to which attention is here called falls, no doubt, for all practical purposes, entirely out of account.

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# *On Spontaneous Phagocytosis, and on the Phagocytosis which is Obtained with the Heated Serum of Patients who have Responded to Tubercular Infection, or, as the case may be, to the Inoculation of a Tubercle Vaccine.*

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(From the Pathological Laboratory of St. Mary's Hospital, London, W.).

(Communicated by Sir John Burdon-Sanderson, Bart., F.R.S. Received October 21,—Read November 23, 1905.)

It has been indicated in the foregoing paper than an *incitor element** is to be found in the blood of those who have made an immunising response to tubercular infection, or, as the case may be, to an inoculation of a tubercle vaccine. This fact does not stand by itself.

### *Recital of Previous Observations on the same Subject.*

The observations of *Metchnikoff*, following in sequence upon the classical researches of R. Pfeiffer on the intraperitoneal destruction of bacteria by the aid of immune sera, first drew attention to the fact that very active phagocytosis comes under observation when bacterial cultures, or as the case may be spermatozoa, are introduced into the peritoneal cavity of normal animals in association with heated serum derived from immunised animals.

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* The term "incitor-element" (Latin—*incito*: I hasten, I urge forward, I bring into rapid movement) is here employed to denote the element in the heated serum which promotes phagocytosis. By employing this term, pending the elucidation of the nature and mode of action of the element in question, we secure the advantage of leaving these issues unprejudged.

*Savtschenko** obtained in experiments conducted *in vitro* with the heated sera of animals which had been subjected to injections of red blood corpuscles, phagocytosis of these formed elements.

*Neufeld and Rimpau*,† working with heated sera derived from animals which had been immunised against streptococcus and pneumococcus, and conducting their experiments *in vitro*, have described these immune sera as possessing a power of inciting phagocytosis. This power was, be it remarked, not numerically measured.

*Leishman*,‡ employing the numerical method for the measurement of phagocytosis which was devised by him with the modifications introduced by one of us in conjunction with Douglas, ascertained that the sera derived from Malta fever convalescents, or as the case may be from men who had undergone anti-typhoid inoculation, retain, after heating, elements which promote phagocytosis.

*Dean*, working with the same methods, without however conforming to the easily realised conditions§ which are essential to the accuracy of the enumeration, has described incitor elements in the heated serum derived from animals which had been immunised against staphylococcus.

Lastly, *Douglas*, employing again the same methods, has obtained evidence of the presence of an incitor element in the heated serum derived from himself and others after inoculation with a sterilised culture of the plague bacillus.

### *Views of the Observers above mentioned on the Nature of the Incitor Element contained in the Heated Serum.*

Influenced by the theoretical conception that the increased resistance to bacterial invasion which is obtained by bacterial inoculation is in every case referable to a modification of the phagocytes,|| Metchnikoff originally spoke of the incitor element as a *stimulin*.

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* 'Annales de l'Institut Pasteur,' 1902.

† Neufeld and Rimpau's paper was published in the 'Deutsche Med. Wochenschrift' in September, 1904, 12 months after the first description of the opsonins in these 'Proceedings.'

‡ 'Path. Soc. Trans.,' 1905, vol. 56.

§ "I should not feel disposed," remarks this author ('Roy. Soc. Proc.,' Series B, vol. 76, p. 511), "to place quite the same reliance as Wright and Douglas on the numerical accuracy of the results which can be derived from their method. Where the leucocytes are very full, *i.e.*, where the counts are high—it is impossible to differentiate results by the method of enumeration." In spite of the perfectly self-evident experimental limitation of our method, which Dean here recognises, this worker employs in practically all his published experiments bacterial suspensions which give him an *average* phagocytic count often of 50 and more bacteria in the leucocyte. Such a count is altogether incompatible with accurate quantitative work.

|| The correctness of the view that artificial immunity depends upon a modification of

This appellation may, we think, be characterised as unfortunate, *first*, because the mode of action of the incitor element was prejudged; *secondly*, because the appellation suggests (in contravention to everything which has come to light with respect to immunisation) that there are elaborated in the animal organism in response to inoculations, not *vaccinotropic* elements (elements which have a chemical affinity for the vaccine) but *leucocytropic* elements (substances which have a chemical action on leucocytes).

At a later date the terms "sensitiser" and "fixing substance" (*la substance sensibilitrice* and *le fixateur*) were applied by Metchnikoff to the incitor element. This nomenclature is, it seems to us, almost equally infelicitous—infelicitous because it imposes upon the mind the following ideas:—(*a*) that the phenomena of phagocytosis are analogous to those of hæmolysis; (*b*) that the incitor substance, like the "amboceptor" of Ehrlich, exerts its specific effect only in the case where it is reinforced by a complement; and (*c*) that the mechanical movements of the phagocyte in the ingestion of particulate matter are analogous to the chemical action of the complement in the case where red blood corpuscles are dissolved by a hæmolytic serum.

With the exception of Leishman,* who, with a view to conforming to the original nomenclature of Metchnikoff, and also because his own experiments incline him to adopt the same point of view, speaks of the incitor substances as *stimulins*, all the other observers† take the view that the

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the leucocytes was first inquired into by Denys and Leclef ('La Cellule,' 1895, vol. 11), in connection with their experiments conducted on rabbits with streptococcus. The doubt with regard to the correctness of Metchnikoff's view which found expression in the paper of these authors was further justified by the experiments of Mennes ('Zeitsch. f. Hygiene,' 1897, vol. 25), conducted with the blood of animals immunised against the pneumococcus. Finally, the incorrectness of the view that immunisation depends on a modification of the leucocytes was for the first time unambiguously established by one of us working in conjunction with Douglas ('Roy. Soc. Proc.,' vol. 72, p. 369, and vol. 73 p. 129). Our results were afterwards confirmed by Bulloch ('Roy. Soc. Proc.,' vol. 75).

* *Loc. cit.* and 'Journ. of Hygiene,' 1895.

† It may be remarked in this connection that Neufeld and Rimpau, while satisfied that the incitor substances in the serum exert an opsonic action on the bacteria, suggest that the term *opsonins* should be here rejected and that the substances here in question should be called *bacteriotropins*. Pending the discussion of the questions of the mode of action of the incitor elements in the heated serum, and of their identity or non-identity with the opsonins found in normal blood, it will suffice here to remark with respect to the proposed nomenclature of Neufeld the following:—

(*a*) The term *bacteriotropins* (since it connotes nothing more than the property of entering into chemical combination with bacteria) is more appropriate as a generic term for the whole class of substances which combine chemically with bacteria, than as a specific designation for the substances which prepare the bacteria for phagocytosis.

(*b*) All considerations of the comparative merits of Neufeld's terminology and my terminology apart—there must, I apprehend, remain to me as the author of the term incitor element in the immune serum exerts an opsonic action upon the bacteria, preparing them for phagocytosis.

### *Sources of Fallacy which must be Eliminated before the Question as to the Nature of the Incitor Element in the Heated Serum can be Properly Investigated.*

Before an inquiry into the nature of the incitor constituent of heated "immune serum" can be properly taken in hand, the sources of fallacy which are incident to such an inquiry must be realised. A *first* source of fallacy is associated with the occurrence of *spontaneous phagocytosis.* A *second* source of fallacy arises, as we shall see in a subsequent section, in connection with the fact that the incitor power of the heated immune serum is influenced in a remarkable and, for the present, quite inexplicable manner by the duration of the exposure to heat and by the temperature employed.

### *Fallacy of Spontaneous Phagocytosis.*

It will enforce itself upon the mind on considering the protocols of the original experiments published by one of us in conjunction with Douglas in these 'Proceedings'* that the phagocytosis is not completely

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*bacteriotropic substances* ('Lancet,' December 23, 1899) as against Neufeld the right of assigning to this term its technical signification.

Dean likewise, while championing the view that the incitor element is an opsonin, and while dissatisfied with the ambiguity of the terms "fixateur" and "substance sensibilitrice," and while conceding that "it may be convenient to adopt the term *opsonin*," employs instead the periphrasis "*the substance which prepares the micro-organisms for phagocytosis*," denying himself the convenience of the term opsonins "in order to mark the danger that one might be led to regard the opsonin as actually a different substance, and not merely a property of immune serum." My fellow-worker, Douglas, and I have not claimed for ourselves anything more than this: that we have, by the aid of an accurate quantitative method, adapted from Leishman, placed in a clear light the *rôle* of the blood fluids in relation to phagocytosis, a *rôle* which was practically everywhere ignored or misconceived, and which had at best been "glimpsed" by one or two observers whose work, undertaken with very defective and fallacious technical methods, was, as Dean's own analysis shows, of a very unconvincing character. We submit that the clarification of the *rôle* of the blood fluids which was effected by us would have remained incomplete and ineffective if we had not alighted on the terms "opsonic power" and "opsonins," or some other apposite and equally convenient nomenclature to denote, as the case may be, the power or "*the substance in the serum which prepares the micro-organisms for phagocytosis.*"

We would also submit that the ultimate—and we hold for the present unapproachable question—as to whether the opsonic effect we have described is only one of a series of diverse effects exerted by a single *antitropic* substance, or whether it is the result of the specific activity of an independent chemical unit in the serum, is not prejudged by the employment of the term *opsonin.*

* Vols. 72, 73, and 74.

abolished by the heating of even a normal serum. The residual phagocytosis registered in the protocols must, as reflection will show, be either *spontaneous phagocytosis,* meaning by this phagocytosis occurring apart from any co-operation of the serum, or phagocytosis dependent upon the chemical activity of an element which has resisted the destructive action of heat.*

When face to face with the consideration that the elimination of all spontaneous phagocytosis must be a necessary preliminary to the proper investigation of every question which has reference to the presence of an incitor element in heated serum, a suggestion from our fellow worker Captain Stewart R. Douglas, I.M.S., led us to inquire whether the phagocytic activity of the leucocyte might not be affected in a conspicuous manner by the salt content of its fluid environment. Captain Douglas's suggestion was a happy one. For, as will appear in the next section, we found that in certain concentrations of salt the leucocytes display considerable spontaneous phagocytosis with respect to the tubercle bacillus, while again in other salt concentrations spontaneous phagocytosis with respect to these micro-organisms is entirely suppressed.

### *Investigation of the Influence of the Salt Content of the Fluid Environment of the Leucocyte upon Spontaneous Phagocytosis.*

The general results of our experiments conducted with tubercle bacilli will be best submitted in the form of the subjoined graphic curves.

In *Curve* 1 we show the phagocytic counts obtained in an experiment conducted without any admixture of serum. In these experiments one volume of washed blood corpuscles, suspended in 0·85 per cent. NaCl solution, was mixed in each case with an equal volume of suspension of tubercle bacilli in distilled water, and with one volume of a graduated solution of salt. It will be observed that the spontaneous phagocytosis which is here in question is greatest where the phagocytic mixture contains 0·6 per cent. of NaCl, and that the count falls off in a gradual manner, and finally reaches a figure which does not differ sensibly from zero when a concentration of 1·2 per cent. NaCl is arrived at.

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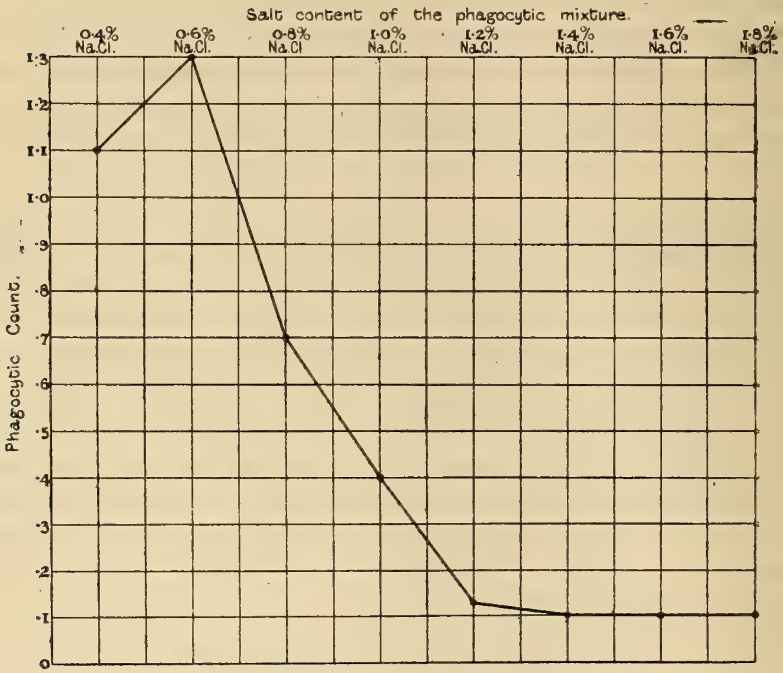
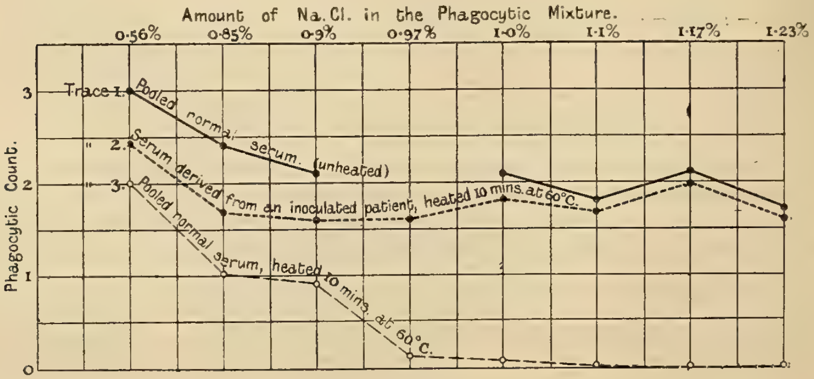
* In favour of the former of these two alternative explanations of the residual phagocytosis is, *first*, the difficulty of conceiving in connection with the experiments conducted with carmine and Indian ink particles that these were chemically acted upon by the serum; *secondly*, the difficulty of explaining, otherwise than as a result of individual differences in phagocytic activity as between leucocyte and leucocyte, the fact that in preparations made with heated normal serum and tubercle bacilli suspended in physiological salt solution, the phagocytosis is generally restricted to a very small percentage of the leucocytes instead of coming into evidence, as in the case of experiments conducted with unheated and active serum, in association with practically all the mature leucocytes.



Curve 1.

In *Curve* 2 we show the phagocytic counts obtained in films prepared from phagocytic mixtures containing a *double* volume of undiluted serum, a *double* volume of washed corpuscles suspended in 1 per cent. NaCl, and a *single* volume of a suspension of tubercle bacilli made in the same menstruum, supplemented in each case by a *single* volume of a solution of sodium chloride of progressively increasing strength.



Curve 2.

Three different sera were here subjected to experiment—

1. The pooled unheated serum derived from eight normal men;

2. The same serum after it had been exposed to a temperature of 60° C. for 10 minutes; and

3. Serum from a patient who had been subjected to therapeutic inoculations of tubercle vaccine. This serum, like the last, had been exposed to a temperature of 60° C. for 10 minutes.

It will be seen that, as in Curve 1, where no serum was employed, the highest phagocytic counts were with each serum obtained where the concentration of the sodium chloride was least.

In the case of Trace 3 (obtained with the heated normal serum) the phagocytosis must be interpreted throughout as purely spontaneous phagocytosis.

In Trace 1 and Trace 2 it must, in the case where low concentrations of NaCl are in question, be interpreted as spontaneous phagocytosis supplemented—to an extent corresponding with the differences between the counts in these traces and those in Trace 3—by phagocytosis dependent upon the chemical action of the serum. Lastly, in these two first traces the phagocytosis registered where high concentrations of NaCl were employed must be entirely dependent upon the chemical action of the serum.

In *Curve* 3 we show the effect of making progressive dilutions of one and the same normal unheated serum with, in the one case a 0·6 per cent. NaCl solution, and in the other case a 1·3 per cent. NaCl solution, using, as in the experiments above, in each case one and the same suspension of tubercle bacilli and one and the same washed blood cream.*

It will be seen that while in the lower trace the phagocytic count sank away in an almost regular manner to zero as the opsonins of the serum were more and more diluted, in the upper trace the phagocytic count increased as the serum was diluted by a less concentrated salt solution.

We do not see room to doubt that in the case of the lower trace spontaneous phagocytosis was completely suppressed, and that such phagocytosis as was obtained was due exclusively to the action of the opsonins, and that in the case of the upper trace the phagocytosis obtained in the outset was due to spontaneous phagocytosis supplemented by the action of the opsonins, while the increased phagocytosis in the latter part of the trace was entirely due to spontaneous phagocytosis.

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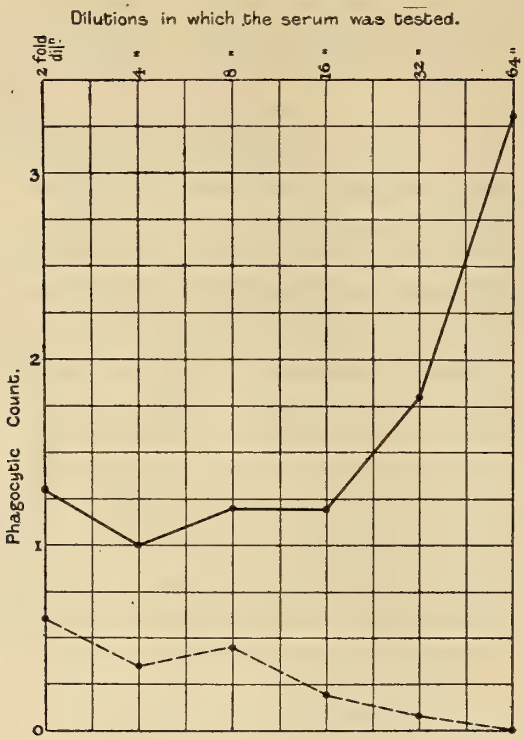
* By this procedure there were obtained in the first case phagocytic mixtures whose salt content diminished from 0·8 to 0·7 NaCl, and in the second case phagocytic mixtures in which the salt content increased from 0·92 to 1 per cent.



Curve 3.

### *Fallacy which may be introduced by the exposure of the Serum for a different period to different degrees of Temperature.*

In view of the research of Dreyer,* which brought out the fact that the agglutinating power of a serum may, as progressively higher temperatures, or as the case may be progressively longer exposures are employed, be first lost and afterwards recovered, it suggested itself that an analogous effect might possibly be exerted upon the incitor element of an immune serum when exposed for different periods to different temperatures.

The results of a typical experiment carried out with such a serum are embodied in the *Curve* 4 below.

It will be seen that while the incitor power of the immune serum was in each case preserved, a very different phagocytic count was obtained according as the serum was exposed to temperatures of 50°, 55° and 60° for a shorter or longer time.

It will be manifest, in view of these results, that where experiments are conducted with heated sera care must be taken to see that in every case the same conditions are observed in the matter of the heating of the serum.

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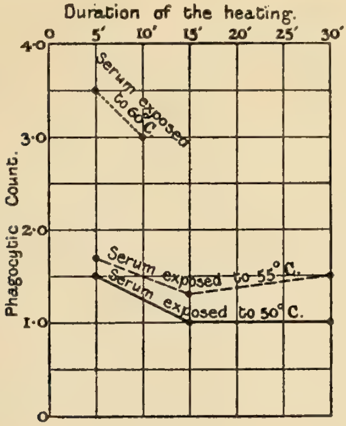
* 'Brit. Med. Journ.,' September 10, 1904.



CURVE 4.

### *Investigation of the Question as to whether the Incitor Substance which is found in the Heated Serum of Persons who have responded to Tubercular Infection, or as the Case may be to an Inoculation of Tubercle Vaccine, is a Leucocytotropic Element—to which the Appellation "Stimulin" would Apply—or Bacteriotropic Element, to which the Term "Opsonin" would Apply.*

The absorption method of Ehrlich, which has already been employed by Neufeld and Rimpau in connection with the investigation of the nature of the incitor element contained in the serum of animals immunised against streptococcus and pneumococcus, was obviously the method indicated for employment in connection with the problem here before us. It was also manifest, in view of the facts detailed in the previous section, that the comparative experiments instituted with heated immune serum before, and after digestion with tubercle bacilli and subsequent centrifugalisation, would yield unfallacious results only on adhering rigidly to the same conditions in the matter of the heating of the serum, and on arranging the experimental conditions in such a manner as to achieve in the phagocytic mixtures employed in each case a salt content of over 1 per cent. NaCl.

A series of experiments conducted with these precautions showed in an uniform manner that the incitor element can be completely extracted from heated immune serum by digestion for half an hour at 37° C. with a suspension of tubercle bacilli.*

It is thus clear that the incitor element which is found in heated serum of persons who have responded to tubercular infection, or as the case may be to the inoculation of a tubercle vaccine, is an opsonin. We may, pending the discussion in the next section of its identity and non-identity with the opsonin of the unheated normal serum, speak of this opsonin in a provisional manner as the *opsonin found in the heated immune serum.*

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* This result is in conformity with the results obtained by Neufeld and Rimpau in connection with streptococcal and pneumococcal immune serum, and by Dean in connection with staphylococcal immune serum.

### *Question as to whether the Opsonin found in Heated Immune Serum is or is not Identical with that found in the Unheated Normal Serum.*

Leishman, who has spoken of the incitor element in the heated immune serum as a *stimulin*, in common with Neufeld, working in conjunction with Rimpau, and Dean, who have shown that this incitor element functions as an *opsonin*, have laid emphasis on the thermostability of the incitor element. Both Leishman and Neufeld urge that the character thermostability differentiates the incitor elements they have in view from the thermolabile opsonins described by one of us in conjunction with Douglas. Neufeld goes further, and contends that the particular opsonins which have been described by him as thermostable alone possess any significance in connection with the protection of the organism against bacterial disease. In support of this contention Neufeld adverts to the fact that man, although he is, according to experiments recorded by one of us in conjunction with Douglas, the possessor of thermolabile opsonins against the plague bacillus, is none the less not protected against this micro-organism.

Before investigating the question of fact as to the identity or non-identity of the opsonins of the normal and immune organism, which are discriminated from each other by Neufeld, we may be allowed to comment on the standpoint which he takes up. We submit that he proceeds upon an entirely erroneous conception when he assumes that the non-immunised human organism does not offer a resistance to such bacterial infections as plague. We submit, further, that it is erroneous to conceive of the normal organism as differing from the immunised organism in a qualitative manner. Rather, does not the theory of Ehrlich brilliantly teach that in immunisation we are never building upwards from a level of absolute non-resistance, but always building upon a foundation which is already laid—calling into existence in increased quantity and conveying into the blood only such chemical agents as exist already preformed in the body?

Reverting from this digression, we may address ourselves to the investigation of the facts, and may inquire whether they plead against or in favour of the identity of the opsonins which are found in the unheated normal blood with the opsonins which are found in the heated immune blood.

In the investigation of the facts we have built upon the following postulates:—

(*a*) If the so-called thermostable opsonins are in reality thermostable, it will make no difference to the result whether the serum is heated in a diluted or in an undiluted condition. If on the other hand the thermostable opsonins represent nothing other than a residuum of thermolabile opsonins which has escaped destruction by heat, it may quite well happen that the serum will be completely inactivated if, before the heat is applied, the serum is adequately diluted.

(*b*) Again, if the serum as derived from an immunised organism contains in its native condition a mixture of opsonins, which are respectively thermolabile and thermostable, we may, in conformity with the all-round greater chemical stability of thermostable substances, expect that the thermolabile opsonins will be destroyed when exposed to sunlight, and that the thermostable opsonins will remain unaffected.

(*c*) Lastly, if the reputedly thermostable opsonins constitute an altogether new and distinct category of opsonins produced in the course of immunisation, we may expect, at any rate in cases where the immunisation has been carried very far, to find the thermostable opsonins greatly in excess of the thermolabile opsonins. In such a case it would be reasonable to expect the heated serum to bear almost as much dilution as the unheated serum before the point is in each case reached where the opsonic power is lost. On the contrary, if the so-called thermostable opsonins represent only an undestroyed residuum of the ordinary thermolabile opsonins, we may expect the heated serum to forfeit its opsonic power by dilution sooner than the unheated serum.

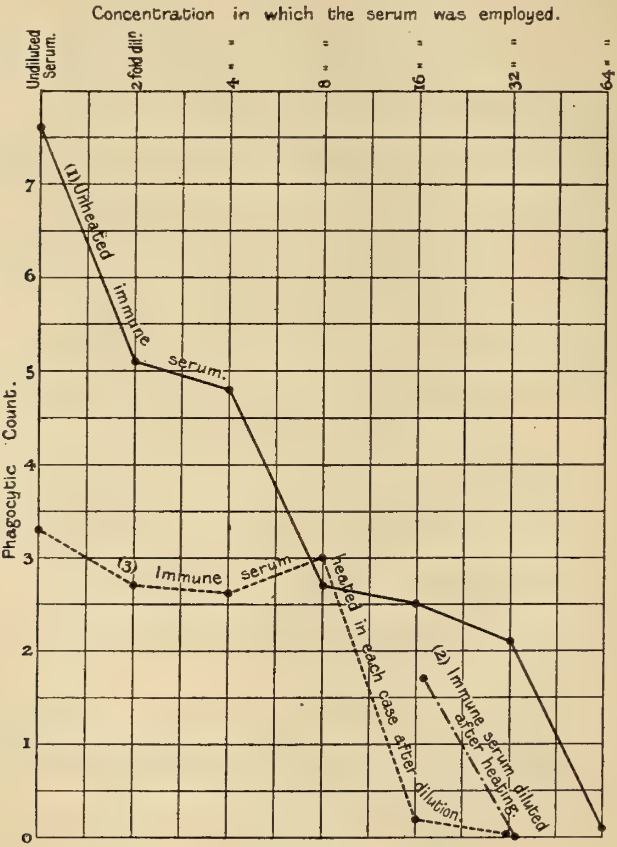
The graphic curves which are subjoined will serve to bring before the eye the results of, in each case, a typical experiment instituted with a view to the resolution of the questions suggested above.

*Curve* 5 furnishes an answer to the questions suggested in (*a*) and (*c*); *Curve* 6 an answer to the question suggested in (*b*).

*Explanation of Curve* 5.—The experiment, whose results are here graphically set forth, had a double object in view. Its first object was to determine whether the tuberculo-opsonic power of the serum derived from an inoculated patient would be only partially abolished in the case where heat is applied to the undiluted serum, and would be completely abolished when heat is applied to the diluted serum. Its secondary object was to determine how far one and the same serum could be diluted before and after heating before its tuberculo-opsonic power was extinguished.

The serum which was employed for the purposes of this experiment was obtained from a patient whose opsonic index had been raised from 0·17 to

1·8 by repeated inoculations of new tuberculin, and who had under the influence of these inoculations completely recovered from tuberculous ulcers of the leg, which had laid bare the tendons, and which had for a period of 13 years previously to the commencement of the inoculation treatment defied all treatment.



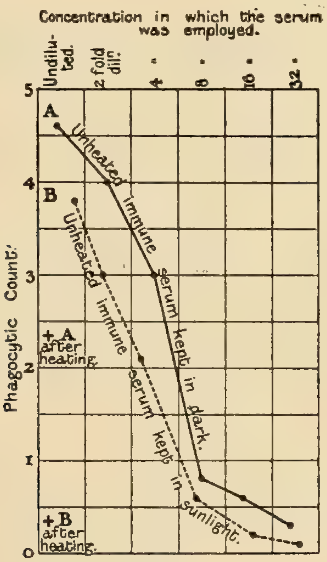
CURVE 5.

So far as the quantity of serum which was available allowed of this being done, these questions were investigated, the precautions explained above being in each case rigidly observed.

It will be seen on reference to the graphic curve, and on comparison of the phagocytic counts registered in the case of the 16-fold dilutions, that while the serum which had been first heated and then diluted (Trace 2) gave a phagocytic count of 1·7, the serum which had been first diluted and then heated (Trace 3) gave a phagocytic count of practically zero. It will further appear on referring to the three traces that, while the opsonic power of the unheated serum was maintained till a 64-fold dilution was arrived at, the opsonic power of two samples of serum which were heated respectively before and after dilution was extinguished when in the former case a 32-fold, and in the latter case a 16-fold dilution was arrived at.

*Comment.*—The experiment shows that the opsonin found in heated serum is destroyed by heat when the serum is sufficiently diluted.*

*Explanation of Curve No.* 6.—In the experiment here in question we employed a serum derived from a patient with tubercular peritonitis, who had responded to infection in a characteristic manner.



CURVE 6.

Dividing it into two portions, we exposed one portion to direct sunlight for a period of six to eight hours, keeping the other portion in the dark in an incubator at 22° C. for the same time.

We now measured the opsonic power of each portion of serum both in the unheated condition and after exposure to 60° C. for 10 minutes. In the case of the unheated samples we tested in each case not only the undiluted serum but also in each case a series of progressive dilutions. In the case of the samples which were heated we tested only the undiluted sera.

It will be seen on comparing the phagocytic counts obtained with the insolated and non-insolated sera respectively, that while in the case of the unheated samples the serum which had been exposed to sunlight gave throughout almost as high a phagocytic count as the serum which had been kept in the dark, in the case of the heated samples the serum which had been exposed to sunlight gave a zero result, while the specimen which had been kept in the dark gave a count of 2·3 bacilli to each leucocyte.

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* Further experiments bearing on this question will be found in the Appendix.

*Comment.*—The experiment shows that the reputedly thermostable opsonin is—in contradiction with what is known to hold of other thermostable elements—eminently heliolabile.

### *Conclusions with respect to the Nature of the Incitor Element which is found in Heated Immune Serum after it has been Exposed to Heat.*

Manifestly the plain teaching of our experiments is, that the opsonin which is found in the heated immune serum of a patient who has responded to tubercular infection, or as the case may be to the inoculation of a tubercle vaccine, does not differ with respect to its resistance to heat and sunlight from the opsonin which is found in the unheated normal serum.

A precisely similar conclusion with respect to the identity of the opsonins found respectively in unheated normal and heated immune sera was, we may note, arrived at by Dean in connection with his experiments on the sera of animals which had been immunised against staphylococcus.

We have only to remark in conclusion that if we prefer to speak of the opsonin as a thermolabile element, and Dean prefers to speak of it as a thermostable element, there is nothing at issue between us except the question as to whether it is in harmony with usage, and with the genius of the English language as employed in scientific discourse, to characterise as "thermostable" an element of which at best residual traces remain in the case of the normal serum where this has been heated to 60° C., and in the case of the immune serum where this has, after adequate dilution, been heated to the same temperature.

## APPENDIX.

It may be convenient to subjoin here, in tabular form, the results of three experiments, similar to that set forth in Curve V, in which the opsonic power of a tuberculo-immune serum was measured in a series of dilutions made in the one case after the serum had been heated to 60° C. for 10 minutes, and in the other case before the serum was so heated.

| Serial number of the experiment. | Source from which the serum was derived. | Phagocytic count obtained in the case of the heated undiluted serum. | Dilution in which the opsonic power was measured. | Phagocytic count in the case where the serum was heated before it was diluted. | Phagocytic count in the case where the serum was diluted before it was heated. |
|---|---|---|---|---|---|
| Expt. 1 ... | Pooled serum of six patients who had been inoculated with tubercle vaccine | 2·4 | 2-fold dilution | 1·9 | 3·3 |
| | | | 4-fold | 2·7 | 1·7 |
| | | | 8- „ | 1·1 | 0·6 |
| | | | 16- „ | 1·0 | 0·45 |
| | | | 32- „ | 0·97 | 0·2 |
| | | | 64- „ | 0·75 | 0·08 |
| Expt. 2 ... | Serum of a patient (E. M.) who had been inoculated with tubercle vaccine | — | 4-fold | 1·5 | 2·7 |
| | | | 8- „ | 1·4 | 1·9 |
| | | | 16- „ | 1·6 | 1·2 |
| | | | 32- „ | 1·5 | 0·3 |
| | | | | 0·9 | 0·05 |
| Expt. 3 ... | Serum of a patient (J. B.) who had been inoculated with tubercle vaccine | 1·4 | 2-fold | — | 1·5 |
| | | | 4- „ | 0·85 | 1·5 |
| | | | 8- „ | 0·7 | 1·6 |
| | | | 16- „ | 0·7 | 0·2 |
| | | | 32- „ | 0·25 | 0·0 |
| | | | 64- „ | 0·0 | — |

# *On the Occurrence of Heterotypical Mitoses in Cancer.*

By E. F. Bashford, M.D., and J. A. Murray, M.B., B.Sc.

(Communicated by J. Rose Bradford, M.D., F.R.S. Received November 2,—
Read November 23, 1905.)

[Plates 5 and 6.]

The present paper refers to a communication* made to the Royal Society in January, 1904. In that paper and its expansion,† published later, we emphasised the significance of the zoological distribution of cancer; we discussed the unique features of the processes responsible for the experimental transmission of carcinoma from one animal to another and the limitations to its successful attainment: we also published a series of figures depicting the characters of the nuclei of cancer cells during division, in the malignant new growths of fishes and mammals. We shall give a different explanation of the mitoses we figured in our earlier communications as resembling the heterotypical mitoses of reproductive tissue. We have found that those mitoses may be interpreted as somatic mitoses with longitudinally split chromosomes. Their apparent heterotypical form is thus due to variations in the development of the achromatic figure, the peculiar form of the chromosomes and their mode of attachment to the spindle.

Our figures of heterotypical mitoses in cancer confirmed the observations of Farmer, Moore and Walker, communicated‡ to the Royal Society at the preceding meeting, but we dissociated ourselves from their conclusions on the diagnostic value and the significance of the phenomenon. The amount of chromatin entering into the equatorial plate of the dividing cells of human cancer had long been known to be subject to diminution (von Hansemann,§ 1893), but the presence of heterotypical mitoses appeared to throw a new light on its occurrence and meaning.

We have pointed out that the characteristic changes accompanying the heterotypical mitosis in the reproductive tissues are absent from cancer cells undergoing what we regarded as this form of division, and that the want of correspondence extends to the stages which precede and follow it.‖ We have also illustrated some of the appearances simulating bivalent chromosomes, but in reality conforming to the type met with in ordinary (somatic) karyokinetic cell-division.* In what follows we shall illustrate other sources of error on the basis of a renewed analysis of the preparations from which the figures of heterotypical mitosis in our previous papers were made and by other figures not yet published.

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* 'Roy. Soc. Proc.,' vol. 73.

† First Scientific Report, Cancer Research Fund.

‡ 'Roy. Soc. Proc.,' vol. 72.

§ 'Studien über die Spezificität, den Altruismus und die Anaplasie der Zellen,' Berlin, 1893, etc.

‖ *Loc. cit.*, and 'Lancet,' April 1, 1905.

In the sexual cells of animals the heterotypical mitosis is preceded by a stage known as the "synapsis." In it the chromatin filament is split longitudinally and gathered into a rosette at one part of the nucleus, the nucleolus lying to one side and usually flattened against the nuclear membrane. In this stage the chromosomes are believed to unite in pairs, thus giving rise in the equatorial plate of the heterotypical mitosis to bivalent chromosomes, half as numerous as those characteristic of ordinary somatic cells. The examination of many sporadic and transplanted malignant new growths failed to reveal a corresponding sequence in their nuclei. We therefore undertook a renewed analysis of the preparations of the stages in cell-division already figured from transplanted mouse tumours, and of other preparations resembling them, to determine whether or not their identification as heterotypical were justified. We shall confine our statements mainly to five transplantable mouse tumours because they permit of control observations with varying methods of preservation and staining in a manner not possible with material from sporadic new growths; but our remarks apply also to the figures we have published from sporadic tumours of the trout and cat.

Von Hansemann† has combated the statements on the presence of heterotypical mitoses in malignant growths, and ascribes the appearances figured to clumping of the chromosomes, and to pathological abnormalities in their form. He adheres to his conclusion that the numerical diminution is not an exact halving of the normal number, but is irregular, and due to (1) asymmetrical mitosis, and (2) casting out and degeneration of chromosomes. Häcker reproduced three of our figures in a paper in which he admitted the striking similarity to heterotypical mitosis, but suggested that adhesion of the chromosomes in pairs,‡ together with longitudinal splitting of the couples thus produced, might account for the phenomena encountered in cancer. The appearances in some preparations are explicable in the manner suggested by von Hansemann,* but all the forms of cell-division resembling the heterotypical mitoses of reproductive tissue cannot be accounted for in this manner.

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* *Loc. cit.*

† 'Biolog. Centralb.,' vol. 24, 1904; vol. 25, 1905; 'Verhandl. physiol. Ges.,' Berlin, 1904.

‡ Häcker uses the term "heterotypical" in a purely morphological sense, and embraces in it the mitoses of the cells destined to give rise to the sexual products of Ascaris and the Copepods. In the latter he has shown that exposure to ether ('Anat. Anzeiger,' 1900) may cause all the cells of the developing egg to exhibit this modification (viz., cohesion of the chromosomes in pairs). In the strict sense of the word, the term "heterotype" is

Some of the nuclear divisions previously figured have been found on re-examination to be due to an artificial grouping together of distinct chromosomes. Fig. 1, reproducing at higher magnification fig. 3 of our Royal Society paper, and fig. 27 of the First Scientific Report of the Imperial Cancer Research Fund, affords an example of this source of confusion. The chromosomes seem to be bivalent, *i.e.*, to have the form of rings and loops. This mitosis is not completely depicted in fig. 1, the remainder of the chromosomes being in the next section. The preparation has been carefully restained. The result is shown in figs. 2 and 3. The "rings" and "loops" resolve themselves into a larger number of ordinary short chromosomes, split longitudinally. The dense equatorial plate of the next section shows clearly the presence of many short chromosomes split longitudinally, and arranged in the manner described below. The mitosis is therefore somatic, and not heterotypical.

The ordinary scheme of karyokinetic cell-division presents, in its phase of equilibrium (amphiaster or equatorial plate), a series of V-shaped loops with limbs of equal length, arranged around a central spindle and all lying in a plane at right angles to its axis. This arrangement is by no means universal. Some of the deviations are of great importance to a proper understanding of what occurs in cancer. Frequently the limbs of the V-shaped loops are of unequal length. When this is the case the attraction fibres are relatively few in number and attached only to the apex of the V and its immediate vicinity, and therefore nearer one end of each chromosome than the other.

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applied to the form of mitosis characteristic of the first ripening division of the spermatocytes of amphibia, as first described by Fleming. In the Salamander, ring-shaped chromosomes, half as numerous as the longitudinally split chromosomes of somatic mitoses, are stretched out into elongated ellipses upon the spindle, giving rise to a barrel-shaped figure. Chromosomes of similar form are associated with their numerical reduction in many animals and plants, but it must be borne in mind that different forms of chromosomes occur in the corresponding mitoses of some animals. In Ascaris, *e.g.*, both ripening divisions appear to be effected by a longitudinal splitting of chromosomes arranged transversely on the spindle, and in others ring or loop chromosomes are never formed. It was, of course, conceivable that reduction-divisions might occur in cancer by means of chromosomes unlike those in the reproductive tissues of the same animal. The frequency of cells with diminished numbers of chromosomes led us to examine many tumours for evidence of their occurrence, but without result.

* 'Biolog. Centralb.,' vol. 24, 1904.

As a consequence the longer limb does not come to a position of equilibrium in the equatorial plane, but may take up one inclined to the axis of the achromatic figure. When the attraction fibres are attached to the chromosomes in this manner in a cell of elongated form, the longer limbs may even come to lie parallel to the spindle axis, and nuclear divisions closely resembling heterotypical mitoses may result. In such mitoses pairs of distinct chromosomes whose longer limbs lie on opposite sides of the equator, while their apices are closely opposed, simulate bivalent chromosomes, *cf.* figs. 5 to 10. In polar view the apparent halving of the chromosome number due to superposition, and the unusual vertical extension of the free longer limbs, are even more deceptive; unless the longitudinal splitting of the chromosomes is very clear, the resemblance to heterotypical mitoses may be almost perfect. During the separation of the daughter chromosomes such nuclear divisions are especially deceptive, because the longer limbs adhere for some time after separation of the apices and short limbs. Barrel-shaped forms result, in which the crowding together of the chromosomes renders their enumeration impossible, but at the same time conveys the impression of a diminution in their number.

A much more serious source of error results from individual differences in the size of the chromosomes in one and the same nucleus. Montgomery* and Sutton† have drawn attention to this phenomenon in the sexual cells of invertebrates. We have found it to be present also in many vertebrate mitoses. When the chromosomes attain the position of equilibrium in the equatorial plate the smaller take up a position nearer the axis of the central spindle than the larger or more massive ones. Seen in profile the larger may then completely screen the smaller from view, and lead to an under-estimate of the chromosome number.

Normally, nuclear division takes place by means of bipolar mitosis distributing the halves of the chromosomes to two daughter nuclei. An interesting abnormality results, when, from any cause, this segregation of the daughter elements does not take place. This may either result from the centrosome, remaining single or, if after division of the centrosome, one only become attached to the chromosomes by the attraction fibres. The chromosomes then remain in one group, but the daughter elements separate slightly from each other before combining to form one large nucleus, containing twice as many chromosomes as the mother nucleus. This form of mitosis, known as a "monaster" (from the presence of only one active attraction sphere) has been studied and described in detail by Th.* and M.† Boveri in invertebrates: figs. 13, 14, and 15, from a squamous cell carcinoma of the tongue (human), represent a cell in this condition. The nuclear membrane has disappeared, but there is no trace of radiations or centrosomes to be seen. The chromosomes are arranged as a hollow sphere around a central clear area, and for the most part consist of two parallel daughter elements in different stages of separation. Some chromosomes in which the separation of the daughter elements is least advanced have a horse-shoe shape. In others, the daughter elements are parallel and widely separated. In a few, the separation is incomplete, the ends only remain apposed, giving the appearance of rings. In fig. 13 this cell has a striking resemblance to a heterotypical mitosis such as occurs in testis. Its cytoplasm is clear and voluminous, and there are no intercellular bridges between it and the surrounding epithelial cells. The number of chromosomes in the cell, however, is not diminished, but amounts to 45 to 50 when both sections are examined. Such a mitosis, instead of reducing the number to half, really results in the original number of chromosomes being doubled, because the separated halves of the chromosomes combine again to form a single nucleus.

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* T. H. Montgomery, 'Proc. Acad. Nat. Sc., Philadelphia,' 1901 ; 'Biol. Bull.,' vol. 4, 1903.

† W. S. Sutton, 'Biol. Bull.,' vol. 4, 1902.

After elimination of these sources of confusion, there remain other apparent heterotypical mitoses which cannot be explained in any of these ways. The chromosomes present an irregular contour and become drawn out like a viscid fluid in the later stages of separation. Individual chromosomes cannot be made out. The achromatic spindle develops at such a rate that the evolution of the chromosomes cannot keep pace with it, and they are drawn towards the spindle and stretched upon it before they have completely contracted and condensed.

All these abnormalities may occur in nuclei possessing the usual number of chromosomes. They also occur in cells with a greater or lesser number associated with the presence of multipolar and asymmetrical mitoses in other cells. In such cases there is no evidence warranting the assumption that the diminution in the number of the chromosomes is due solely to a nuclear division effecting a reduction comparable to that of the sexual cells.

Galleotti‡ and von Hansemann§ have shown that nuclei with diminished numbers of chromosomes (hypo-chromatic) may arise from larger ones by asymmetrical mitosis, in which entire chromosomes pass to one daughter cell, because they are only attached to one or other attraction sphere, and also by "casting out of chromatin." "Casting out of chromatin" is merely an exaggeration of what occurs in asymmetrical mitosis; in it some chromosomes remain unattached to either attraction-sphere, and therefore fail to be included in either daughter nucleus. Krompecher* and we ourselves† have shown that multipolar mitoses may also lead to a diminution in the number of chromosomes. We stated that nuclei with diminished and half the somatic number of chromosomes occur without it being possible to determine whether the diminution has been effected by asymmetrical mitosis, casting out of chromatin, multipolar or heterotypical mitosis.

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* Th. Boveri, 'Sitz.-ber. Phys.-med. Ges.,' Würzburg, 1897; 'Zellenstudien,' vol. 4, Jena, 1901.

† M. Boveri, 'Jen. Zeitschr. f. Naturwiss,' vol. 37, 1903.

‡ 'Ziegler's Beitr.,' vol. 14, 1893.

§ *Loc. cit.*

We have given our reasons for now believing that the mitoses we formerly assumed confirmed the occurrence of a heterotypical reducing division in cancer, are, in reality, somatic mitoses. Although we do not presume to explain in the above manner all the figures which may be brought forward resembling that form of nuclear division, we submit that the occurrence of heterotypical mitoses in cancer requires further proof. Multipolar mitosis and other irregular forms of cell-division occur in cancer, but they do not supervene upon heterotypical mitosis. They are entirely independent of its presence, and, of themselves, suffice to account for the diminutions frequently occurring in the number of chromosomes in cancer throughout the vertebrates.

## DESCRIPTION OF PLATES.

[Plates 5 and 6.]

Fig. 1.—Apparent heterotypical mitosis. Transplanted carcinoma of mouse. Analysis of ring, loop, and bivalent chromosomes (heterotypical). Replica of fig. 3 of Royal Society paper, and of fig. 27, First Scientific Report, 1904. × 3000/1.

Fig. 2.—Same section as fig. 1. Analysis after restaining, showing how a fortuitous association of short somatic (longitudinally split) chromosomes gives the appearance of bivalent elements. × 3000/1.

Fig. 3.—Partial analysis of the remainder of the mitosis, of which part only is shown in figs. 1 and 2. Longitudinally split chromosomes with limbs of unequal length lying at various angles to the spindle axis. × 3000/1.

Fig. 4.—Diagram of a somatic amphiaster, in which longitudinally split V-shaped chromosomes, with limbs of unequal length, are apparently arranged parallel to the spindle axis. Adjacent chromosomes, with their longer limbs on opposite sides of the equator, if regarded as together forming one chromosome, would convert such a mitosis into a heterotype with half the somatic number of chromosomes arranged longitudinally on the spindle, *e.g.*, figs. 3, 5, 6, 7, 8, 9, and 10.

Figs. 5 and 6.—Apparent heterotypical mitosis. Fig. 5, replica of fig. 4, Royal Society paper, and of fig. 26 in First Scientific Report, 1904. Transplanted carcinoma of mouse. Chromosomes arranged longitudinally on the spindle. The mitosis is contained in two consecutive sections. × 3000/1.

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* 'Centralb. f. Path. u. path. Anat.,' vol. 13, 1902.

† *Loc. cit.*

FIGS. 7 and 8.—Same sections as figs. 5 and 6. Result of analysis after restaining. Longitudinally split chromosomes with unequal limbs projecting above and below the equatorial plane. × 3000/1 of diagram, fig. 4.

FIG. 9.—Apparent heterotypical mitosis. Transplanted carcinoma of mouse. Loop and figure-of-8 chromosomes arranged longitudinally on the spindle. × 3000/1.

FIG. 10.—Analysis of the same preparation as fig. 9, showing the slight differences in interpretation sufficient to make this mitosis conform to the somatic type. The loop chromosome in the middle of the equatorial plate consists of two distinct V-shaped chromosomes with unequal limbs projecting above and below the equator. The attraction fibres are attached to the apices, and not to the ends of the long limbs as would be the case in a true heterotype. × 3000/1.

FIG. 11.—Diagram of a somatic metaphase in which the limbs of the chromosomes are of unequal length. The longer limbs still cohere after separation of the apices and shorter limbs. The barrel-shaped figure thus produced resembles a heterotype, especially when the compressed form of the cytoplasm crowds the chromosomes together.

FIG. 12.—Shows the detailed analysis of the mitosis at the upper part of fig. 20, Plate 7, Second Scientific Report, 1905. It illustrates the mode of separation of daughter chromosomes with unequal limbs, as represented diagrammatically in fig. 11. Transplanted carcinoma of mouse. × 3000/1.

FIG. 13.—Microphotograph (untouched) of "monaster" mitosis from squamous-celled carcinoma of the tongue (man). Shows ring and U-shaped chromosomes. × 1000/1.

FIG. 14.—Analysis of same section as fig. 13. Partial separation of the daughter chromosomes accounts for the presence of ring and U-shaped chromosomes. No centrosomes or achromatic figure visible. × 3000/1.

FIG. 15.—Remainder of same cell in next section. Shows large number of chromosomes of ring and U-shape, along with others in which the widely separated daughter-rods are parallel to each other. × 3000/1.

*Bashford and Murray.* *Roy. Soc. Proc., B. vol. 77, Plate 5.*

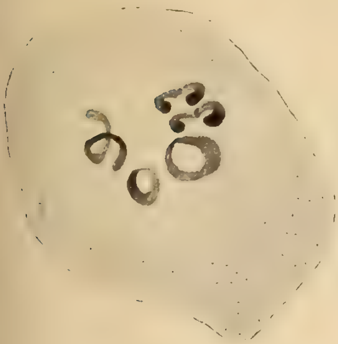


Fig. 1.

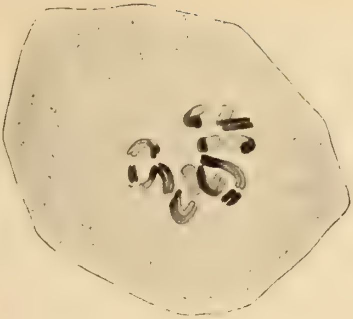


Fig. 2.

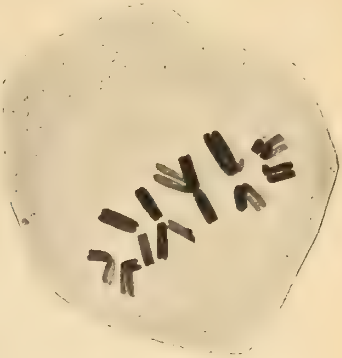


Fig. 3.

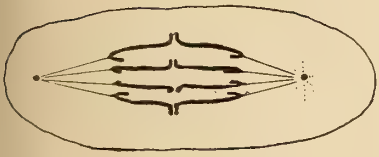
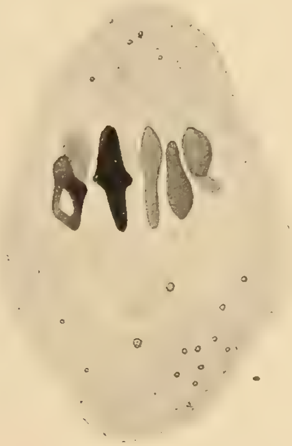


Fig. 4.



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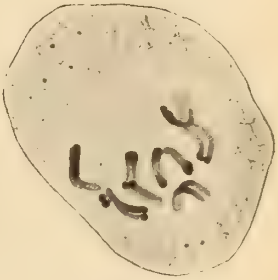

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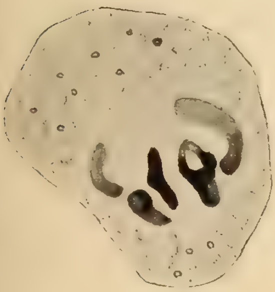

Fig. 5.

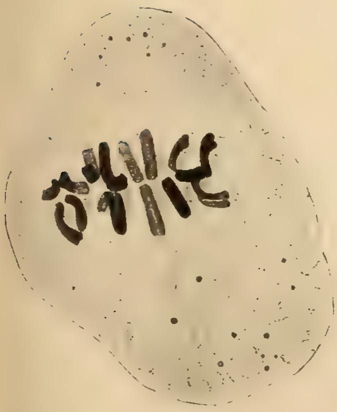

Fig. 8.

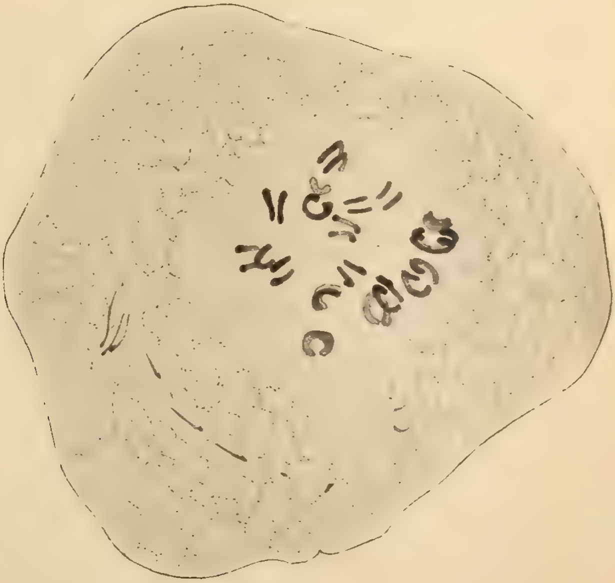

Fig. 15.

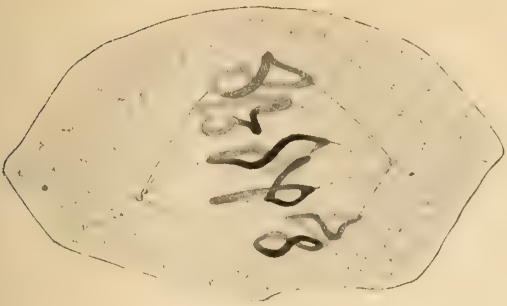

FIG. 9.

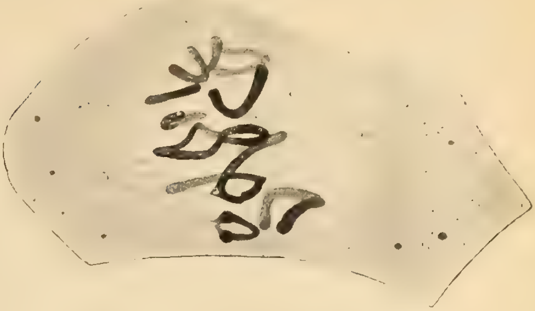

FIG. 10.

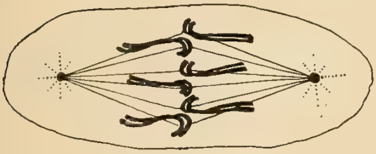

FIG. 11.

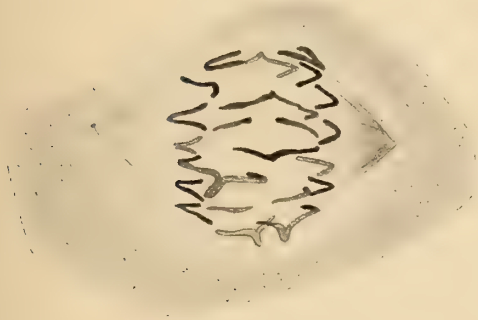

FIG. 12.

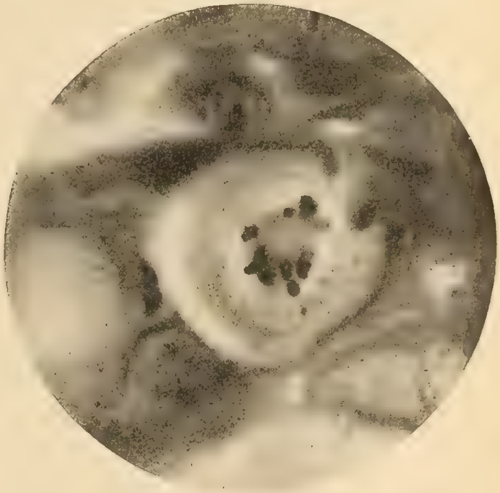

FIG. 13.

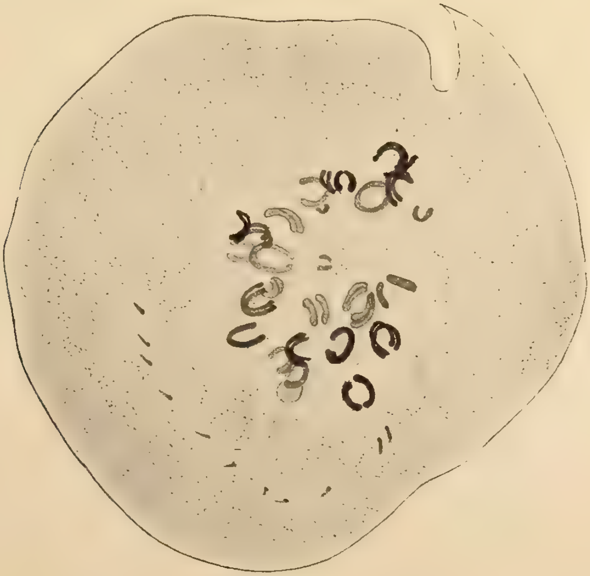

FIG. 14.

# *Pathological Report on the Histology of Sleeping Sickness and Trypanosomiasis, with a Comparison of the Changes Found in Animals Infected with* T. Gambiense *and other Trypanosomata.*

By ANTON BREINL, M.U.Dr. (Prague), J. W. Garret International Fellow, Liverpool School of Tropical Medicine. University, Liverpool.

(Communicated by Professor R. Boyce, F.R.S. Received April 8,—Read May 11, 1905.)

Three cases of Sleeping Sickness and one case of Trypanosomiasis dying in Liverpool have been histologically examined. The central nervous system of the Sleeping Sickness cases showed the changes described by different observers, Mott, Low, the Portuguese Commission and others. One case exhibited an intra-pial hæmorrhage of the spinal cord, extending from the sixth cervical segment to the third thoracic segment, about 7 mm. broad. In another case there occurred four larger hæmorrhages, besides numerous smaller ones, in the grey substance, chiefly affecting the posterior cornua and the thoracic part of the cord. Microscopically the brain and spinal cord showed small celled infiltration around the vessels, consisting for the most part of lymphocytes, some plasma cells and phagocytes, between which were a varying number of red cells in different stages of disintegration. The intima of the vessels showed a proliferation of the endothelial cells. Red and white blood corpuscles were often seen in the vessel walls. Here and there the blood vessels were filled with white blood corpuscles resembling a thrombosis.

It is most striking that the small celled infiltration is much more marked in the grey substance of the nervous centres, especially in the large grey ganglia, than in the peripheral parts. Very numerous capillary hæmorrhages of different sizes were present in these situations. Infiltration around the vessels of the membranes and in the tissues of the pia and arachnoidea was observed. Around the infiltrated vessels degeneration of the fibres and an excess of glia cells were seen, sometimes exhibiting the picture of red softening. The ganglia cells showed an irregularly distributed degeneration, central and peripheral chromatolysis and also partial pyknosis.

Signs of inflammation and small celled infiltration in the endo- and perineurium of the peripheral nerves were seen. In two cases of longer duration and with more pronounced symptoms of the disease, more definite changes around the vessels of the brain and spinal cord were seen than in the third case of shorter duration with less marked symptoms. In this case the perivascular changes in the brain were small and were still less so in the spinal cord.

In all groups of the lymph glands numerous ones were found showing the typical appearance of hæmo-lymph glands with a pronounced hyperplasia of the connective tissue, a widening of the follicles and the formation of a system of sinuses containing red blood cells and large phagocytes in a fine threadwork of connective tissue. Others showed a transition between the hæmo-lymph glands and normal glands, one part appearing normal, the other presenting a typical sinus formation with numerous red blood cells and phagocytes. Nearly all the glands contained between the lymph cells a number of blood corpuscles, many in all stages of degeneration. The spleen was greatly congested and contained a few necrotic areas, scattered through the organ was a little blood pigment giving the iron reaction. The bone marrow exhibited the typical picture of red marrow with gelatinous degeneration. The liver and kidneys showed hæmorrhages between the parenchyma cells, which latter appeared to be undergoing degeneration. In all three cases a few large bacilli and cocci were seen which did not stain by Gram's method, these I consider to be due to *post-mortem* contamination.

The bacteriological cultivation, anaërobic and aërobic of the cerebro-spinal fluid and the blood of two cases, did not give any growth, and moreover, animals infected with large quantities of cerebro-spinal fluid or blood did not show any other symptoms than those caused through the presence of trypanosomes in the blood.

In only one case, dying with a fair number of trypanosomes in the blood, could I find occasionally a parasite in the congested vessels of the organs.

The one case of trypanosomiasis, which died from an intercurrent pneumonia, did not show any other changes in the central nervous system than the very large peri-vascular spaces, partially filled with transudate, and sometimes containing a few white blood corpuscles. The ganglion cells showed the changes corresponding to the hyperthermia. The lymph glands were very hæmorrhagic, some showed the typical appearance of hæmo-lymph glands.

The brains, spinal cords, and organs of numerous animals infected with *Trypanosoma Gambiense*, monkeys, rabbits, guinea-pigs, dogs, rats, and mice were examined. One of the monkeys showed a typical hæmorrhagic cicatrix in the left *lobus centralis* of the brain; other monkeys and a chimpanzee showed a high congestion of the vessels of the brain and spinal cord, with hæmorrhages, around the vessel walls, containing lymphocytes, a few leucocytes, and phagocytes. The intima showed large proliferated endothelial cells, the vessels often contained very many leucocytes. Numerous hæmorrhages in the grey substance of the spinal cord were frequently seen. Some of the dogs, rabbits, and guinea-pigs showed the changes in the spinal cord, and to a less extent in the brain. The ganglion cells exhibited similar alteration as in the human cases. In some of the animals no changes around the vessels and very little alteration of the ganglion cells and fibres were noted.

Many of the lymph glands presented the picture of hæmo-lymph glands with a few pigment granules; sometimes an irregular patchy appearance was seen, the centre consisting of a light stained area with numerous red cells and phagocytes, the periphery of normal lymph tissue with a small number of follicles. The spleen showed congestion in the more acute cases, with irregular hyperplasia of the malpighian bodies, in the older cases hyperplasia of the connective tissue. For comparison the brains, cords, and organs of animals infected with *T. dimorphum* (Gambian horse disease) were examined. In a few cases the same hæmorrhages as described above and localised in the grey substance of the nervous centres were seen. The lymph glands showed the peculiar appearance; as noted above the light spaces were completely filled with blood pigment. The spleen showed hardly any pigment. Trypanosomes were found mostly clumped together in the vessels of the different organs of all animals dying with numerous parasites in the peripheral blood.

### *Conclusions.*

(1) In the cases of Sleeping Sickness there is a pronounced congestion of the blood vessels of the central nervous system together with a small celled infiltration around the vessels of the brain and spinal cord, especially in the grey substance.

(2) Chromatolysis and pyknosis of the ganglion cells of brain and spinal cord.

(3) Inflammation of the leptomeninges of the brain and spinal cord.

(4) Neuritis of the peripheral nerves.

(5) The more chronic the case and the more pronounced the symptoms the greater the changes in the brain and cord.

(6) The majority of the lymph glands exhibit the picture of hæmo-lymph glands.

(7) Small necroses of the spleen and signs of degeneration of the bone marrow.

(8) The brain of a case of Trypanosomiasis did not show small celled infiltration.

(9) Animals infected with *Trypanosoma Gambiense* show sometimes changes in the nervous system, localised in the grey matter, hæmorrhages, lymphocytes, and a few leucocytes in the peri-vascular space: hæmo-lymph glands in large numbers, and sometimes necrosis of the spleen and degeneration of the bone marrow.

(10) Animals infected with *Trypanosoma dimorphum* exhibit similar changes in the nervous system and organs. A far greater deposit of pigment in the lymph glands and in older cases in the spleen is present.

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(10) Animals infected with *Trypanosoma dimorphum* exhibit similar changes in the nervous system and organs. A far greater deposit of pigment in the lymph glands and in older cases in the spleen is present.

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## *Further Experiments on Inheritance in Sweet Peas and Stocks: Preliminary Account.*

By W. Bateson, F.R.S., E. R. Saunders, and R. C. Punnett.

(Received December 1,—Read December 7, 1905.)

Later results have provided expressions which include many of the peculiar phenomena of inheritance already witnessed in sweet peas and stocks. In sweet peas we have shown that purple may occur, as a "reversion," from the cross between two whites, one having long pollen grains, the other round. Similarly in stocks, white glabrous × cream glabrous gives "reversionary" $F_1$ purple hoary. (In both cases the parents are whites, *i.e.*, free from sap-colour, for cream is due to yellow plastids, recessive to colourless plastids.)

The appearance of coloured flowers is due to the simultaneous presence in the zygote of two factors, belonging to distinct allelomorphic pairs, which may be spoken of as C, *c*, and R, *r*, the large letter denoting presence, the small letter the absence of the particular factor.

Hoariness of stocks is similarly due to the coexistence of two other factors, and the presence of either of these factors is also allelomorphic to its absence. These two pairs are spoken of as H, *h*, and K, *k*. But, though H and K may both be present, no hoariness is produced unless C and R, the colour-factors, are also both present. For the actual development of hoariness four factors are thus required. The existence of white-flowered hoary plants creates a difficulty; but white *incana* is evidently a coloured form in reality, for its flowers tinge on fading, and its embryo has the deep-green colour characteristic of purple varieties. Apart from breeding-tests, however, white hoary *Bromptons* show no visible indication of colour, and as yet they constitute a marked exception.

White glabrous and cream glabrous types contain both H and K, the two elements of hoariness. One of them contains C and the other contains R. All sap-coloured types studied contain one only of the two factors H, K. Consequently, we find the following result, which formerly seemed paradoxical:—

| | | | | $F_1$. |
|---|---|---|---|---|
| 1. | Cream glabrous | × | Red or purple glabrous | Red or purple hoary. |
| 2. | White glabrous | × | Ditto | Purple hoary. |
| 3. | Cream glabrous | × | White glabrous | Ditto. |
| 4. | Any red or purple glabrous. | × | Any red or purple glabrous. | Red or purple glabrous. |

The truth of this account appears from the fact that in $F_2$ from cream glabrous × white glabrous all the coloured are hoary and all the whites are glabrous. Again, purple (hoary) *incana* × cream glabrous gives in $F_2$ all the hoary plants *coloured*, and all the glabrous plants *white*; while "white" (hoary) *incana* × sap-coloured types gives in $F_2$ coloured hoary, coloured glabrous, and in addition tinging "whites" in both classes.

When a character is produced by the meeting of factors belonging to two distinct allelomorphic pairs, the $F_2$ ratio will be 9 : 7 (*i.e.*, $3+3+1$), and consequently, when in sweet peas and stocks a coloured $F_1$ is produced from two non-sap-coloured types, the $F_2$ ratio is 9 coloured : 7 white; but there are 4 gametically-distinct types among the coloured and 5 among the whites. Most of these have been now recognised experimentally.

When $F_1$ is purple the coloured class consists of purples and reds. In both sweet peas and stocks the ratio is 27 purple, 9 red, 28 white, composed thus:—

$$27 : 9 : \underbrace{9 : 9 : 3 : 3 : 3 : 1}_{28}.$$

The purples are due to the presence of a "blue" factor B, allelomorphic to *b*, its absence. Unless C and R are both present, B cannot be perceived without breeding tests. The three pairs, C, *c*, R, *r*, B, *b*, by entering into all possible combinations according to the simple Mendelian system, give the results observed.

This scheme takes no account of the sub-classes which sometimes occur in both purples and reds. Several of these are merely superposed on the primary classes, while others are more complex and require further analysis. The distribution of the colours shows further complications when some coloured strains were introduced as original parents.

"Reversion" is thus seen to be a simple and orderly phenomenon, due to the meeting of factors belonging to distinct though complementary allelomorphic pairs, which at some moment in the phylogeny of the varieties have each lost their complement.

*Pollen-characters in Sweet Peas.*—Gametic coupling of a novel kind exists in this case. The whole generation in $F_2$ consists of 3 long : 1 round. The whites taken alone also are 3 long : 1 round. But in the purples there is a great deficiency of rounds, while in the reds they are greatly in excess. This result indicates that there is a partial coupling of the long pollen-character with the factor B, and a corresponding coupling of round pollen with *b*. This peculiarity only occurs in families which contain *both* purple and red members. The gametic output of $F_1$ in these cases is approximately

$$7AB + 1Ab + 1aB + 7ab,$$

where A is long, and *a* round pollen. This arrangement gives a close approach to the observed figures:—

| | Purple. | | Red. | | White. | |
|---|---|---|---|---|---|---|
| | Long. | Round. | Long. | Round. | Long. | Round. |
| Observed ...... | 1528 | 106 | 117 | 381 | 1199 | 394 |
| Calculated ...... | 1448·5 | 122·7 | 122·7 | 401·5 | 1220·5 | 407·4 |

# *Report on the Psychology and Sociology of the Todas and other Indian Tribes.*

By W. H. R. Rivers, M.D., Fellow of St. John's College, Cambridge.

(An Abstract of Work carried out by the aid of the Gunning Fund of the Royal Society for the year 1901–1902. Communicated by the Secretaries of the Royal Society. Received October 18,—Read December 14, 1905.)

Six months were spent in India, the greater part of the time being devoted to the investigation of the Todas of the Nilgiri Hills. The senses of these people were examined experimentally on the same lines as those followed by the Cambridge Expedition to Torres Straits.* The general result was to confirm the chief conclusion of this expedition that there are no great differences between the senses of savage and civilised races. In pure sense-acuity little difference was found, and the observations lend no support to the view that the sense-acuity of savage or barbarous races is superior to that of civilised man, the apparent superiority in some cases being due to the training of observation in special directions.

In two senses only is there distinct evidence of difference between Todas and Englishmen in sensory endowment. The Todas are distinctly less sensitive to pain than the average educated Englishman, and they show the same kind of deficiency in the colour-sense which has been found in other races of low culture, especially in the Papuan † and the Egyptian peasant.‡

The Todas are distinctly less sensitive to blue than the average educated Englishman, though differing little in sensibility to red or yellow. This defect in the sensibility for blue is associated with the deficient nomenclature for this colour which is almost universal in races of low culture; and the observations on the Todas strengthen the conclusion reached by previous work that physiological insensitiveness is one, though only one, of the factors upon which the defect in language depends.

The most striking feature of Toda colour-vision, however, is the great frequency of colour-blindness. About five hundred individuals were tested, and over 12 per cent. of the males were found to suffer from typical red-green blindness, the proportion in European races being about 4 per cent. In most races of low culture colour-blindness is less frequent than in

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* 'Reports of the Cambridge Anthropological Expedition to Torres Straits.' Cambridge, vol. 2, Part I, 1901, and Part II, 1903.

† *Loc. cit.*, p. 48.

‡ 'Journ. Anthrop. Inst.,' 1901, vol. 31, p. 229.

Europe, but the Todas show the highest recorded frequency of this condition in any race. By means of the genealogies preserved by the Todas the relationship between the colour-blind people could be traced, and a body of material was obtained which illustrates the mode of hereditary transmission of the defect.

The Todas were found to be subject to various geometrical-optical illusions; and quantitative observations were made on the illusion of compared vertical and horizontal lines and on the Müller-Lyer illusion. The Todas are subject to the former in a greater degree than English observers, and to the latter in a smaller degree. The two illusions differ in nature: the former is probably largely physiological in origin, and is neutralised by the experience of civilised life, while the latter is more strictly psychological in character; and the different reaction of the Todas to the two illusions is in accordance with this difference in their nature.

In every measurement the degree in which the individuals of each race differed from one another was studied; and a mass of material was collected for the study of variability in the reaction to psychological tests, and for the analysis of the complex conditions upon which the coefficients of variation depend.

On comparing the observations of Todas, Papuans, and Englishmen, all tested by the same methods, and chiefly by the same experimenter, it is found that there is some evidence of a correlation between the degree of general intellectual development and certain simple mental properties or activities which can be tested by experimental methods. In general intellectual development the Todas occupy an intermediate position between Papuans and Englishmen, and a similar intermediate position is occupied by them in connection with many of the tests.*

The social and religious institutions were also studied. The sociology was investigated largely by means of the genealogical method,† and the system of kinship, the complex marriage regulations and the laws of inheritance and property were worked out in detail.

The Todas were found to possess a highly elaborate religious ceremonial of which only brief sketches had previously been published, while many ceremonies had wholly escaped observation. In consequence, much time was devoted to the detailed investigation of this ceremonial and of the other features of the Toda religion. Evidence is given that this religion is one which has undergone degenerative changes, and some evidence is advanced in favour of a view that the Todas are a people who have once had a culture higher than that they now possess. When the customs and institutions of the Todas are compared with those of other parts of India, it is found that there is most resemblance with the people of Malabar; and the view is advanced that the Todas migrated to the Nilgiri Hills from Malabar, and are possibly allied in race to the two chief castes at present existing in that district, the Nairs and Nambutiris.

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* A full account of the senses of the Todas will be published shortly in the 'British Journal of Psychology,' vol. 1, Part IV.

† 'Journ. Anthrop. Inst.,' 1900, vol. 30, p. 74.

In addition to the work on the Todas, observations were also made on members of other tribes. The vision of the Sholagas and Uralis, two wild jungle tribes, was investigated* from several points of view; and observations, chiefly on colour-blindness, were made on members of other castes or tribes.

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# *A Study of the Process of Nitrification with reference to the Purification of Sewage.*

By Harriette Chick, D.Sc.

(Communicated by Professor H. Marshall Ward, F.R.S. Received April 1.—Read May 11, 1905.)

*Introduction.*—That nitrification is a biological process was first established with certainty, after long controversy, in 1888, by the decisive experiments of Plath† and Landolt,‡ who in this matter confirmed the previous researches of Schlösing and Muntz,§ Warington‖ and Soyka.¶

The discovery of the active living agents followed soon after, when Winogradsky** (1890 to 1892) isolated the two sets of organisms which, as he showed, co-operate to produce natural nitrification. These were (1) the nitrite-producer, *B. nitrosomonas*, which oxidises ammonia to the nitrite stage only, and (2) the nitrite-producer, *B. nitrobacter*, which carries on the oxidation to nitrate but cannot act upon ammonia, being indeed inhibited in its development by minute traces of that substance. Winogradsky, by himself, and in conjunction with Omeliansky,* subjected these bacteria to a very exhaustive study. The most striking characteristic that they demonstrated was the marked repugnance of both forms to organic substances. Not only, in opposition to the rest of the plant world, do these organisms make no nutritive use of sugars, peptones, etc., but the presence of more than a trace of such organic substances was found to entirely inhibit their development, thus explaining the failure of all attempts to isolate these bacteria by using the ordinary nutrient culture-media. Winogradsky, on the other hand, had succeeded in cultivating them by employing a silica-jelly-medium impregnated with inorganic salts, and a total inability to grow on organic nutrient media was afterwards put forward by him as a definite practical criterion of the purity of cultures of nitrifying bacteria. This criterion has been challenged by Burri and Stutzer,† by Stutzer and Hartleb,‡ and later by Fremlin.§ It has been shown by Winogradsky,‖ and also by Gärtner, Fränkel, and Krüger¶ that the former workers were misled by an admixture of non-nitrifying organisms. The most recent work, that of Boulanger and Massol,** and of Wimmer†† confirms Winogradsky's criterion.

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* 'Bull. Madras Government Museum,' 1903, vol. 5, p. 3.

† Plath, 'Landw. Jahrbücher,' v. H. Thiel, vol. 16, hft. 6, and 'Centralbl. f. Agrikulturchem. v. Biedermann,' vol. 17, 1888.

‡ Landolt, 'Deutsch. Landw. Presse,' vol. 15, and 'Centralbl. f. Agrikulturchem.,' vol. 17, 1888.

§ Schlösing and Muntz, 'Comptes Rendus,' vols. 84 and 85, 1877, and vol. 89, 1879.

‖ Warington, 'Journ. Chem. Soc.,' vol. 33, 1878, and 'Landw. Versuchsst.,' vol. 24, 1880.

¶ Soyka, 'Zeitschr. f. Biologie,' vol. 14, 1878.

** Winogradsky, 'Ann. de l'Inst. Past.,' vol. 4, 1890, and vol. 5, 1891: also 'Archives des Sci. biol. de St. Petersb.,' vol. 1, 1892.

The special case of nitrification considered in this paper is that occurring during sewage purification, which aims at the complete oxidation and mineralisation of putrescible substances present. Nitrification is here of great importance, and the effluent of perfectly-treated sewage should contain all its nitrogen in the form of nitrates.

Although land-treatment of sewage is theoretically the most economical, yet artificial processes, by which space can be saved, have often to be employed. Two processes concern us here, both involving the use of "filter-beds" of coke or other porous material, in which the sewage, usually after having been treated in a "septic tank" is oxidised by bacteria. (1) *Contact Filters.*—In these the filter-bed is first entirely filled up with the liquor and then after a time allowed to empty slowly, and finally to remain empty for a period. This cycle usually occupies about eight hours, and often may have to be repeated before the effluent is sufficiently purified. (2) *Continuous*

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* Winogradsky and Omeliansky, 'Centralbl. f. Bakt.,' 2 abt., 5, 1899.

† Burri and Stutzer, 'Centralbl. f. Bakt.,' 2 abt., 1 and 2, 1895 and 1896.

‡ Stutzer and Hartleb, 'Centralbl. f. Bakt.,' 2 abt., 2 and 3, 1896 and 1897.

§ Fremlin, 'Journal of Hygiene,' vol. 3, 1903.

‖ Winogradsky, 'Centralbl. f. Bakt.,' 2 abt., 2, 1896.

¶ Gärtner, Fränkel, Krüger, 'Centralbl. f. Bakt.,' 2 abt., 4, 1898.

** Boulanger and Massol, 'Ann. de l'Inst. Past.,' vol. 17, 1903.

†† Wimmer, 'Zeitschr. f. Hygiene,' vol. 48, 1904.

*Filters.*—In this procedure the liquor trickles continuously through the filter-bed, being uniformly distributed by sprinklers, while as perfect aëration as possible of the bed is maintained.

The objects of the present research were mainly the following:—

1. The detailed chemical study of the course of the nitrification occurring during the filtration of sewage, especially during the maturing period of the filter, and the comparison of the "contact" and "continuous" methods (Section I).

2. The isolation and study of the organisms concerned, and comparison with those isolated from the soil by Winogradsky. The amount of organic matter accumulated in a sewage filter is comparatively great, and it seemed most unlikely that nitrification should here also be the work of bacteria so extremely sensitive to the presence of organic matter. One seemed compelled to believe that other and different bacteria must be here engaged (Section II).

3. The study of the question of absorption of ammonia upon the surface of filtering material previous to nitrification (Section III).

These researches were begun in Vienna in 1901, and were resumed in Munich in 1903, after a break of two years. I am very happy to have this opportunity of thanking Professor Max Gruber for his kind hospitality extended to me in the hygienic institutes of both cities, as well as for the valuable advice and kind assistance he constantly gave me in the course of the work. My thanks are also due to the Royal Commission on Sewage Disposal for granting me leave of absence in 1903 to continue the research in Munich. I should also add that part of the expense of the work was defrayed by a grant from the Royal Society.

## Section I.—*Chemical Study of Nitrification in Experimental Filters.*

*Description of Apparatus and Methods of Analysis.*—Small experimental filters were erected, consisting of glass cylinders 50 cm. high and 12 cm. in diameter; these were placed one above the other, fitted well together by means of specially ground rims, and covered on the outside with black glazed paper. There were altogether three filters, differing only in height—200 cm., 100 cm., and 50 cm. respectively. Fig. 1 is a diagram of the filter of medium height, showing the arrangements made to allow of samples being drawn off, and of the temperatures being measured at different depths; the tall filter, consisting of four cylinders, had the three upper ones similarly constructed. The filters were filled with small coke, carefully sifted and of a uniform size (mean diameter 3·5 mm.). By volumetric measurements with water, it was found that when this coke is packed into a space, the volume of the interspaces between the pieces is 35 per cent.; the volume of the pores inside the pieces (amount of water retained on draining) is 20 per cent.; and the volume of solid coke is 45 per cent. of the whole space. The three filters were fixed to the wall, near together, and all treated in exactly the same way, *i.e.*, as continuous filters. Each received 4 litres of liquid daily, and the sewage employed was the liquid manure ("Jauche") from a neighbouring cowshed; this proved a very suitable material, after a rough filtration through glass wool, and dilution to 1 in 20 with tap-water.

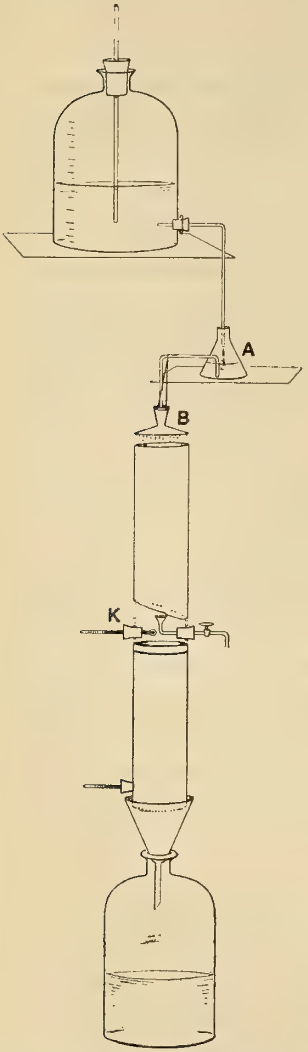

FIG. 1—Diagram of the "Continuous" Filter, of medium height, showing construction. The contour of the filter is represented as interrupted at K, to show the arrangement of the thermometer and the collecting funnel.

This liquid was contained in a 10-litre reservoir bottle of the Mariotte type, from which it dropped regularly into a small vessel containing a siphon arranged to empty when 100 c.c. had collected (A, fig. 1) into a sprinkler (B, fig. 1), the object of which was to distribute the liquid as evenly as possible over the surface of the filter.

The methods of analysis employed in following the course of the oxidation of nitrogen were those usually adopted in such work,* but the following details may be given:—

In the estimation of *free and saline ammonia*, 1 to 10 c.c. of the liquid was taken and diluted in a retort with about 500 c.c. $NH_3$-free water, and distilled, it being found unnecessary to add any alkali.† Three successive portions of 50 c.c. were distilled off and the ammonia they contained estimated by means of Nessler's reagent. *Albuminoid ammonia* was afterwards estimated by adding to the same retort a definite amount of "alkaline permanganate solution," distilling as long as ammonia came over in the distillate, and estimating these amounts in the same way.

*Oxidised nitrogen* in the filtrates was detected by means of the reaction with diphenylamine sulphuric acid. Nitrites were distinguished by reactions with acidified starch-zinc-iodide solution, and with metaphenylene-diamine, and estimated by the use of the latter. When both nitrites and nitrates were present, they were estimated together by the *indigo* method (*Tiemann-Gärtner's* "*Wasseranalyse*") and the figure for nitrates obtained by subtraction. This method was afterwards given up in favour of the *copper-zinc couple* method,* where, to allow for traces of ammonia originally present in the solution, or introduced during analysis, a control estimation was always made; this control was carried out in every way like the real analysis, except that no couple was introduced, and the ammonia obtained was subtracted from that found in the actual estimation.

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* *Cf.* 'Report of Royal Commission on Sewage Disposal,' vol. 4, part 5.

† 'Sew. Com. Report,' vol. 4, part 5, appendices 3 and 4.

*Total nitrogen* was estimated in the sewage by Kjeldahl's method, a small amount of $Na_2SO_4$ only being added during the preliminary heating.

The *oxidisability* ("Oxidirbarkeit," or measure of oxidisable substances present) was estimated by reduction of permanganate in alkaline solution on boiling for ten minutes, care being taken to keep the external conditions (such as concentration, size of flask, total amount of liquid present, temperature, and time of reaction) constant in all determinations. Periodic examination of sewage and filtrate in this way gave useful comparative results and a means of following the course of the general oxidation.

*Course of General Oxidation During Maturation: First Appearance of Oxidised Nitrogen.*—The filters were started on February 20, 1901, and their action carefully controlled by means of analysis from that time onwards. Especial attention was paid to the period of maturation from the time of first using to that of full efficiency, as it was thought this should throw light generally upon the manner of their working. The sewage employed contained on an average:—total nitrogen 10 parts, organic matter (by evaporation and ignition) 21 parts; oxidisability (expressed in terms of O) about 11 parts per 100,000 by weight. The course of the oxidation will be seen by reference to Tables Ia, Ib and II. On March 7 (see Table II, analyses 1 to 4) there was a marked amount of general oxidation taking place in all three filters, or at least a reduction of oxidisable substances, but there was no trace of oxidised nitrogen in the filtrates nor was there any diminution in ammonia. Oxidised nitrogen first appeared in the tall filter on March 18 (the filtrate being then clear, bright and without smell), three days later it was detected in the medium filter and seven days later in the short filter. During this first period (after starting and before the occurrence of nitrification) the amount of ammonia in the sewage was frequently compared with that in the filtrates and found to be always the same.† On March 7 this was proved to be so for all three filtrates, and even on March 28, when the first trace of oxidised nitrogen appeared in the filtrate from the short filter there was no diminution of the free and saline ammonia coming through (*cf.* analyses 10 and 13). So, generally, Tables II and III seem to show that loss of ammonia goes hand in hand with production of oxidised nitrogen.

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* Sutton, 'Vol. Analysis,' 8th ed., p. 452.

† As the sewage varied considerably in composition from time to time, care was always taken that the sewage and filtrate analysed for comparison should correspond to one another as nearly as possible.

Tables IA (Vienna) and IB (Munich) give a clear idea of the general progress of the oxidation of nitrogen. They are compiled from analyses made from time to time, and while the times given for the later stages are approximate, those for the first stage are exact, being based on almost daily tests.

*The Nitrite Stage.*—After the first appearance of oxidised nitrogen in the filtrates, nitrification went ahead, and, in the case of the tall filter, five days later there was nitrous nitrogen in the filtrate about equal in amount to the total nitrogen going on. The two stages in which nitrification occurs were well separated in time, and show very distinctly, first, the production of nitrites in quantity without nitrates, and finally the complete oxidation to nitrates, nitrites being absent. For example, in the case of the tall filter, five weeks after having been started (Table II, analyses 10 and 11) the sewage contained 17 parts ammoniacal and albuminoid nitrogen, which in the filtrate was reduced to 1·5 parts, while 10 parts nitrogen were present as nitrites, nitrates being altogether absent. Three days later a similar result was obtained (analyses 14 and 15), eight weeks later nitrates were being formed in small amount, and an analysis of the filtrate made four months after starting (analysis 21) showed a complete oxidation of the nitrogen, nitrates being present in quantity unaccompanied by nitrites. In the case of the short filter the process was much slower, for an analysis made after four months showed production in the filtrates of nitrites only (analysis 23). This comparative lack of efficiency may be referred to the lower temperature* of the short filter, for an analysis on the same date of the liquid from No. 1 tap of the tall filter, 50 cm. from the top (Table III, 6 and 7) showed the presence of nitrates in abundance, and only traces of nitrites.

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* An exactly parallel fact was noticed with regard to the time of the first appearance of oxidised nitrogen in these two filters. Strictly speaking, the top section of the tall filter should have been exactly comparable with the short filter, and the only possible explanation is that, as the first division of the latter filter stood 150 cm. higher in the room, the discrepancy was due to a temperature difference. Regular observations of temperature had been made by means of the thermometers in the filters, and the higher position in the room was found to be constantly from 1·5° to 2·5° C. higher than the lower one, the temperature of the interior of the filters differing hardly at all from that of the surrounding air. This explanation is confirmed by the results of the Munich experiment, where all the six filters were arranged so that their tops, and not their bottoms, were on one level; hence the first divisions of all were at a similar temperature, and it was found that the first oxidation of nitrogen was observed after the same period in all the three filters of each set.

This complete separation of a nitrite from a nitrate stage is doubtless due to the comparatively strongly ammoniacal nature of the sewage employed. Previous observers*† have shown the inhibitive effect of ammonia upon nitrate-production, and it is probable that during the earlier stages of the maturing period the nitrate bacteria were unable to become established in the filter, and only later, when the ammonia of the sewage was being rapidly oxidised to nitrites, was the environment suited to their growth and development. The sewage employed frequently contained more than 15 parts free and saline ammonia per 100,000, a concentration which has been shown† to be sufficiently high to check completely the production of nitrates in pure culture. An interesting confirmation of this explanation was obtained in the maturing of the Munich filters, where nitrates appeared in the filtrates very soon after the first appearance of nitrites. There was here no such "nitrite stage," and the sewage was much less ammoniacal (2 to 4 parts ammoniacal nitrogen per 100,000).

*Difference of Function in Different Strata of the Filters.*—An attempt was made to study the course of the oxidation at different depths in the tall and medium filters (see Table III). In analysis 3 the "oxidisability" was taken as a criterion and the decrease in the first 50 cm. of the tall filter was found to be almost as great as in the whole length of the filter. One may therefore suppose that the mechanical deposition of suspended particles as well as the absorption of the more complicated organic matter in solution takes place principally in the upper layers of the filter. It is also apparent from analysis 4 that the formation of nitrites (these analyses were made during the nitrite stage) did not at that date take place in quantity in this upper layer but lower down for the most part; this same fact is also shown in analysis 2. In the latter case the free and saline ammonia was also estimated, and the decrease, which is so marked as the sewage passes through the filter, was found not to begin until after the first 50 cm. were passed. The same phenomenon appears in analysis 8 and is a striking instance of the principle, already alluded to, and discussed at length in the section devoted to absorption, that the disappearance of ammonia and the oxidation of nitrogen are closely associated both in time and space.

*Comparison of Contact and Continuous Filters—Munich Experiments.*—From the mature Vienna filters attempts were made to isolate the nitrifying organisms, but before much progress had been made the work was discontinued and was not again resumed until after two years. This second time, in Munich, in 1903, fresh filters had to be matured, and a second opportunity was afforded for studying the maturing process, and nitrification generally.

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* See footnote **, p. 242.

† Warington, 'Chem. Soc. Journ.,' vol. 35, 1879, and vol. 59, 1891.

Filters like those previously described were again erected, and three of them treated as formerly with a continuous trickle of diluted liquid manure. Another similar set of three filters was treated, for contrast, as contact filters by the procedure mentioned on p. 242. Eight litres of sewage were treated every 48 hours by each of the filters. The capacities of the tall,* medium and short filters were respectively, after wetting, 6, 4 and 2 litres, so that each filling remained in contact at least four hours in the two taller filters and two hours in the short one. For 38 out of the 48 hours of the cycle the filters were empty or emptying. These contact filters were cone-shaped below, with a narrow opening that could be closed with a cork for the purpose of filling them.

In the case of the continuous filters, oxidised nitrogen again made its first appearance in the filtrates four weeks after starting (compare Tables Iᴀ and 1ʙ). The fact that all three Munich filters behaved alike in this respect (forming a contrast to the Vienna ones) has already been explained as a temperature effect, see p. 246. It was noticed that during the maturing period the two different stages of nitrogen oxidation merged one into the other, and were not so clearly separated as was the case in the Vienna filters (Tables Iᴀ and Iʙ); noticeably there was here no long period in which nitrites were formed in quantity without any accompanying nitrates. This difference has already been discussed (p. 247), and explanation is doubtless to be found in the much less ammoniacal nature of the sewage here employed.

The contact filters did not yield nearly such good results as the continuous filters (Table Iʙ). The period which elapsed before nitrogen oxidation was apparent was, in the former, more than half as long again as in the latter. Again, when the short continuous filter showed complete oxidation of its nitrogen, the tall contact filter still showed presence of nitrites in its filtrate.

The Munich continuous filters had completely matured in about ten weeks from the time of starting, but they were yielding a very satisfactory effluent much earlier. After three months the sewage was changed for a much more strongly ammoniacal liquid (cows' urine, diluted 1 in 100, containing 14 to 17 parts ammoniacal nitrogen per 100,000), in order to test the capabilities of the filters as regards nitrogen oxidation. Most satisfactory results were obtained (Table II, analyses 33 to 36, and Table IV), the filtrates contained, as a rule, only traces of ammonia and nitrites, but abundance of nitrates. Attempt to further tax the capabilities of the filters met with failure. Cows' urine diluted only 1 in 50, and containing about 30 parts ammoniacal nitrogen per 100,000, was put through the medium filter for about a fortnight, and also at a later date through all three filters, but it was found that they were incapable of oxidising so concentrated a liquid, and the quality of the filtrates deteriorated (*cf.* Table IV).

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* In these Munich experiments the tall filters, both contact and continuous, were only 150 cm. high, instead of 200 cm., as in Vienna.

Throughout the history of these filters there was a considerable loss of total nitrogen from the sewage while filtering through, but it was specially noticeable during the period when the diluted urine was being treated, when in some cases not much more than half the original nitrogen was present in the filtrate (Table II, analyses 33 to 36). This loss is doubtless due to an escape of free nitrogen, set free possibly by decomposition of ammonium nitrite, a very probable intermediate product in the nitrification of ammonia ($NH_4NO_2 = 2H_2O + N_2$). This loss of nitrogen was not so marked in the case of the Vienna filters (Table II, analyses 1 to 23), though it occurred later to some extent. These differences are probably due to the absence or presence in quantity of the organisms involved.

The Munich continuous filters in their later history, and they were worked for about a year, possessed an efficiency rarely met with in large scale filters, showing that this type can give excellent results in the absence of much suspended matter.* The larger filters could not be considered to be heavily worked, but the short filter, which had a capacity of two litres and treated four litres of sewage daily, approximated more nearly to a practical installation. It oxidised daily about 0·5 gramme nitrogen, and this result must be considered extremely satisfactory when the high nitrogenous concentration of this special sewage (14 to 20 parts of ammoniacal nitrogen per 100,000 instead of the 3 to 8 parts usual in ordinary sewage) is kept in mind. The quantities of nitrate appearing in the filtrates from these filters have rarely, if ever, been obtained in practice on the large scale.

## Section II.—*Bacteriological Investigations.*

*Enumeration of Nitrifying Bacteria in the Filtrates.*—It was thought worth while to attempt to count the numbers of nitrifying bacteria† present in the filtrates from the filters, as it was conceivable that such enumerations might furnish a bacteriological criterion of the quality of sewage effluents.

The practical utility of this procedure is, however, diminished by its slowness, due to the sluggish growth of the organisms and to the extremely small numbers of them introduced in the higher dilutions. The method might, perhaps, give useful comparative results even without allowing the maximum time for development in the subcultures, and the rate of growth could be accelerated by a temperature of 28° to 30° C.

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* This can be removed in practice by a preliminary screening or septic tank treatment.

† The recent work of C. C. Frye ('Report of Roy. Com. on Sewage Disposal,' vol. 2, 1902, p. 9) has experimentally verified the view which has been generally held, though doubted in some quarters, that all the nitrification taking place in sewage filters is the work of living organisms, and none of it purely chemical.

The enumerations were made as follows:—The filtrate was successively diluted with sterile water to a tenth degree six times; of these six dilutions (viz., 1 in 10, 1 in 100, 1 in 1000, 1 in 10,000, 1 in 100,000, 1 in 1,000,000) 1 c.c. was used in every case for inoculation into bouillon and into Winogradsky's ammonia and nitrite-containing media respectively,[*] which were distributed in test-tubes, each containing about 5 c.c. The ammonia tubes were subsequently tested for production of nitrites with acidified starch-zinc-iodide solution, and the nitrite tubes for nitrates with diphenylamine sulphuric acid (after evaporation to dryness with $NH_4Cl$ if any nitrite remained unoxidised).

The numbers in which the nitrifying bacteria are present are surprisingly large (see Table V), and it will be seen that there is no strict relation between the numbers present respectively of nitrite and nitrate producers; but the latter would appear to be present generally in less amount even when the filtrate shows complete oxidation of its nitrogen to nitrates. The filtrates used were in all cases from the Munich continuous filters.

*Isolation of the Nitrite Producer.*—The isolation of a nitrite-producing bacterium in pure culture was found to present considerable difficulty and many unsuccessful trials were made. Attempts were first made to isolate it directly from the coke of the filters by the method of dilutions. This method, originally invented by Lister, was formerly employed by Warington[†] and P. and G. Frankland[‡] for the same purpose, but with only partial success. The method here employed was similar to that used by the Franklands, except that much higher dilutions were made, and a larger number of tubes (about 200) containing appropriate culture media[§] were sown with small amounts of liquid from the higher dilutions, bouillon tubes being also similarly inoculated as controls. Repeated attempts to isolate from the coke of the filter were only partially successful.[||] A culture was, however, obtained which was comparatively, but not absolutely, pure; this was used for further isolation experiments, and will be referred to as culture "*a*."

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[*] Omeliansky, 'Centralbl. f. Bakt.,' 2 abt., 5, 1899.

[†] See footnote, p. 247.

[‡] P. and G. Frankland, 'Phil. Trans.,' B, vol. 181, 1890.

[§] Culture solutions used were diluted urine and Winogradsky's solutions.

[||] Starting from such very impure material, the dilution method does not give an adequate return for the great labour it entails. The filtrates might have proved better original material, as in them the nitrifiers sometimes predominated (see Table V).

Culture "*a*," and all cultures showing production of nitrites* invariably contained in quantity a small oval bacillus or coccus, which was recognised as the nitrite-producing organism.

Attempts to obtain a pure culture were further made with the use of ammonium agar as medium,† but without success. The plate cultures showed vigorous formation of nitrites,* but all nitrifying subcultures were found to be impure. The employment of a similar medium composed of agar and diluted cows' urine was equally unsuccessful.

Ordinary gelatine plate cultures were made and bouillon was inoculated from the impure cultures; none of the colonies separated from the former were able to nitrify, although 40 were investigated. From the growths in bouillon, plate cultures were also made on nutrient gelatine and agar, and 70 of the organisms separated were further investigated, but in no case did nitrification occur. This seemed to show that the nitrifying organisms in filters resembled those of Winogradsky very closely. Therefore, in order to decide if the nitrifying organism was or was not able to live in the bouillon, an ammoniacal medium was directly inoculated from the growths in bouillon. Usually there was no nitrite-production (*e.g.*, Table VI, culture "*a*"), and indeed the oval bacillus could in no case be traced in the bouillon growths. In one instance, however (Table VI, culture "*b*"), inoculation from a bouillon growth led to nitrification, but this property was lost after a second generation in bouillon; it therefore seemed probable that, if the nitrifier had not been killed in the bouillon, it certainly had not been able to multiply there.

It thus was evident that, contrary to expectation, the nitrite-producing organisms of sewage filters were also unable to grow upon media containing organic matter; recourse was then had to silica plate cultures, which were made and inoculated according to the directions given by Omeliansky.‡ This operation was accomplished with comparative ease if the original sodium silicate was quite pure; the study and isolation of the separate colonies was, however, found to be exceedingly difficult. The sub-cultures obtained were to all intents and purposes pure cultures, showing pure pictures of an oval, almost spherical organism, resembling the nitrosomonas of Winogradsky, except that it seemed to be somewhat smaller in size. It appeared constantly in the form of zooglœa embedded in the particles of magnesium carbonate at the bottom of the culture tubes, and it stained easily and well. The individual bacteria were often found to be well separated in a culture, but an actively motile stage was not observed.

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* The test for production of nitrites was usually made by allowing a little of the culture fluid, withdrawn with a sterile pipette, to drop into a small quantity of acidified starch-zinc-iodide in a porcelain dish. This was preferred to the similar test with diphenylamine, partly because of its specific nature, and partly because the ferric salt present in the sediment of the culture-tubes also yielded a slight blue colour with diphenylamine.

† $(NH_4)_2SO_4$, 2·0 gr.; NaCl, 2·0 gr.; $K_2HPO_4$, 1·0 gr.; $MgSO_4$, 0·5 gr.; $MgCO_3$, in excess; agar-agar (purified by washing, Beyerinck's method, 'Centralbl. f. Bakt.,' vol. 19, 1896), 20 gr.; distilled water, 1 litre.

‡ See footnote, p. 250.

These cultures, however, still gave a growth, though extremely slow, in bouillon, and this consisted of the other quite inconspicuous organisms present. By means of the dilution method, pure cultures were obtained which yielded absolutely no growth in bouillon when preserved indefinitely either at 37° or at the room temperature. These pure cultures were not, however, robust, and they nitrified very feebly; attempts are now being made to obtain vigorous pure cultures.

*Isolation of the Nitrate-producer.*—The dilution method was also employed for the isolation of the nitrate organism, the original material being a culture obtained during the enumeration experiments (Table V), which showed active oxidation of nitrites. A culture was separated which consisted of the nitrate bacterium mixed with one other species, and the combination, referred to in future as culture "*d*," formed a very interesting symbiosis. Pure cultures were obtained from culture "*d*" by making surface plate cultures, in great dilution, on nitrite agar.* These pure cultures showed a small non-motile bacterium, agreeing in essentials with Winogradsky's organism, though somewhat larger in size. It was a bacterium very thick in comparison with its length, so that it often appeared to be almost a coccus; stains were badly taken up, and it frequently appeared imperfectly and irregularly stained. These pure cultures rapidly changed nitrite to nitrate, when growing in nitrite-containing medium,† the nitrite present being sometimes completely oxidised in less than two weeks. Bouillon on the other hand remained indefinitely sterile; the tubes were kept under observation for seven weeks without there being any sign of growth.

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* Omeliansky, 'Centralbl. f. Bakt.,' 2 abt. 5, 1899.

† Winogradsky's nitrite culture solution was invariably employed, and the cultures were tested from time to time for the production of nitrates. When time enough had elapsed and all nitrite had disappeared, then, on testing the culture liquid, a negative reaction with starch-zinc-iodide, and a positive with diphenylamine proved the presence of nitrates. But if all nitrite were not oxidised, the remainder was decomposed by evaporating to dryness with a little $NH_4Cl$, and the residue dissolved in water and tested for nitrates with diphenylamine. This method has been shown to be quantitative when such substances as sugar and peptone are present (Frankland, 'Journ. Chem. Soc.,' 1888), and it is possible it might also prove a useful method of estimating nitrates in presence of nitrites in sewage effluents.

Pure cultures were also obtained with more difficulty directly from less pure material, by means of nitrite agar plates, but the organism isolated was in every case the same.

All attempts to isolate a nitrate organism by means of ordinary nutrient agar and gelatine were unsuccessful. In no instance was nitrite oxidised to nitrate by any organism separated on such plate cultures, though over 40 such organisms were investigated.

*Experiments with "Symbiotic" Cultures of the Nitrate-producer.*—Although nitrobacter, when alone, is incapable of growing in bouillon, it would appear to be capable of surviving an inoculation into bouillon if not alone, but growing with certain other bacteria. A very instructive set of experiments was made with culture "*d*" (Table VII), in which this strain was inoculated into bouillon through four generations. From each set of tubes nitrite medium was inoculated, and it was found that the change to nitrate occurred invariably in the tubes sown from the earliest bouillon generation, and in two instances also from those sown from bouillon of the fourth generation (Table VII, $d_3$ and $d_4$). The quantities inoculated were large, one or two drops, but it is impossible to believe that nitrobacter would still be present in a fourth generation if no multiplication had taken place in the bouillon. Pure cultures of the nitrate-producer showed no such effects; inoculated bouillon remained quite clear; examined under the microscope it showed complete absence of bacteria, and nitrite tubes inoculated from the bouillon in no case showed any oxidation to nitrate. In Table VIII are shown the results of further experiments in which three pure and four mixed cultures were compared in this respect, and one is compelled to conclude, in explanation, that the presence of the accompanying organism in some way protects the nitrate bacterium from adverse influences present in the bouillon, which it is unable to withstand if alone. Without further experiment, any attempt to explain in what this action really consists must be pure conjecture, but it is possible that the harmful organic substances present are in some way altered by the accompanying organism, and it would be interesting to see whether the nitrate organism in pure culture could thrive in bouillon previously exhausted by its companion.*

Phenomena which present an interesting analogy with these observations are found in the case of certain anaerobic organisms, one instance of which has been precisely investigated by Winogradsky, viz., that of *Clostridium Pasteurianum.*† This strictly anaerobic species was found to be capable of growing aerobically when, and only when, associated symbiotically with a certain aerobic organism which removed the surrounding oxygen and created an oxygen-free environment for it. Such symbioses of various grades must be frequent in Nature where the "pure culture" is almost unknown. The part played by the artificial pure culture in the progress of bacteriology has, of course, been enormous, yet its possibilities are limited, and one must look to the investigation of regulated simple symbioses for a nearer approach, in the laboratory, to the workings of Nature.

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* The experiment was not made in this instance because the cultures had then been isolated a considerable time and their properties were enfeebled.

† 'Archives des Sciences biolog. de St. Petersb.,' vol. 3, 1895.

A break of two years occurred during the course of these investigations. After they were again resumed, Dr. Schultz-Schultzenstein* published the results of bacteriological investigations, having the same aim as the present work. He isolated two kinds of nitrifying organisms from the material of coke sewage filters at Karolinenhöhe, near Charlottenburg, which corresponded exactly to those isolated from the soil by Winogradsky, and no other nitrifying organisms were found. His researches must be regarded as the first published successful attempt to investigate the organisms concerned with nitrification during the artificial purification of sewage, and the results are entirely confirmed by the present investigation. In spite of this anticipation of my identification of these bacteria, I have thought it worth while to describe my isolation experiments in detail, because in a subject of such technical difficulty the experience of an independent worker may be of use to others.

## Section III.—*Absorption of Ammonia and Ammoniacal Compounds during Sewage Purification.*

It has been held that a most important preliminary to nitrification, both in the soil and in sewage filters, is to be found in an absorption of ammonia and ammonium compounds upon the surface of the particles of soil or of filtering material respectively. In the case of the soil, a long controversy has taken place as to whether a physical or a chemical process was here in question,† and the former view, maintained notably by Liebig‡ and his school, has on the whole prevailed. This "adsorption" of ammonia plays an important part in the current doctrine of the action of sewage filters, which considers that nitrification could not take place in the short time taken by the liquid to pass through the filter, and that the nitrites and nitrates appearing at any particular time in the filtrates are the result of a slower change which has been effected by the nitrifying bacteria upon ammonia previously absorbed in some physical manner upon the surface of the filtering material. The procedure for the purification of sewage used in "contact beds" has been held to assist successively the processes of adsorption of ammonia and nitrification. Dunbar and Thumm* consider that, in the "filling" and "full" stages, putrescible and oxidisable substances are retained upon the surface of the filtering material, and are subsequently oxidised at times when the bed is full of air, the oxidation being the work of bacteria in the bed, among which the nitrifying bacteria rank high in order of importance.

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* Schultz-Schultzenstein, 'Mitt. a. d. Kön.-Prüfungsanst. f. Wasservers. u. Abwässerbeseit,' 1903.

† Way, 'Agric. Soc. England Journ.,' series 1, vols. 11—13, 1850—1852, and Mayer, 'Lehrbuch der Agrikulturchemie,' 1871. Lemberg, 'Zeitschr. d. deutsch. geol. Gesellsch.,' vol. 28, 1876.

‡ 'Liebig, 'Ann. Chem. Pharm.,' vol. 94, 1855, and vols. 105 and 106, 1858.

As regards the complex oxidisable putrescible substances of high molecular weight, the solid suspended matter will be retained, of course, by mechanical filtration, while the soluble constituents may, doubtless, be supposed to undergo some physical adsorption.†‡ But the greater part of the nitrogen present in sewage is there in the form of free and saline ammonia, and these are the compounds most markedly retained as the sewage passes through the filter; yet for such simple compounds as these, adsorption by solids has been shown to take place only to a small degree or not at all.§ To attempt to explain removal of ammonia by adsorption then, would appear inadequate.

Special experiments were therefore made to investigate the behaviour of filtering materials with ammonia and its salts; also during the investigations in Section I, careful note was also made of any facts which should tend to confirm or refute the theory of nitrification quoted above.

If the theory of a previous ammonia absorption and a subsequent oxidation were true, then contact beds should be much more efficient nitrifiers than continuous filters, but the contrary proved to be the case, the latter doubtless owing their greater efficiency to their more perfect aëration. Again, while complicated organic substances appeared to be absorbed in the top layer of the filter (Table III, 3), the disappearance of free and saline ammonia was shown usually to take place lower down in the filter (Table III, 1), and to be always associated with the appearance of oxidised nitrogen (Table III, 2, etc.). Moreover, during the maturing of the filters, before oxidation of nitrogen had occurred, no absorption of ammonia could be detected, although this was frequently looked for. It was possible, however, that, at the very first, ammonia had been taken up by the filtering material, to saturation point, and that afterwards no more absorption was possible until nitrification had begun. Unfortunately no analyses were made at the very beginning, but this gap was afterwards filled by special experiments with clean sterile coke.

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* Dunbar and Thumm, 'Beit. zur Abwasserreinigungsfrage,' 1902.

† Soyka, 'Archiv f. Hygiene,' vol. 2, 1884.

‡ Kattein and Lübbert, 'Gesundheitsingenieur,' vol. 25, 1903.

§ Weppen, 'Ann. d. Chem. u. Pharm.,' vol. 55, 1845, and A. Mayer, 'Lehrbuch d. Agrikulturchemie,' 1871.

The tendency of these observations was thus in opposition to any theory of ammonia-absorption by the filtering material, and this opposition was confirmed by the following experiments, made to test the power of various solids to absorb ammonium salts. The solids employed were barium sulphate, sand, and ground-up "clinker," and the experiments were carried out as follows:—

A small quantity of a solid (1 gramme or 2 grammes), previously carefully purified, was weighed out into a small flask, which was then exhausted to remove air films, which might cause imperfect contact of solid and liquid.* A measured quantity (50 c.c. to 100 c.c.) of ammonium chloride solution was added through a tap-funnel, and the whole left standing for 24 hours. The clear liquid was then drawn off by a pipette, and the ammonia estimated in a small portion (1 to 5 c.c.); this was first diluted to about 500 c.c. with $NH_3$-free water, and then distilled and nesslerised. The remaining liquid was well shaken up and the muddy residue analysed similarly: a correction had to be made for the volume of the solid, which was measured, after centrifugalisation, in a graduated tube. In every case, as a control, a blank experiment was also made, similar in every detail except that $NH_3$-free water replaced the ammonium chloride solution. The solids had previously undergone a careful purification by washing, and often, by ignition also. The ammonium chloride solutions were exceedingly dilute, so as to approximate to the concentration of ammonia in ordinary sewage.

In Experiments 1 to 3 (Table IX) the ammonia yielded, both by the clear and the muddy portions of the liquid, was found to have diminished. It was therefore supposed that boiling was insufficient to drive off any ammonia which might have been absorbed by the solid; accordingly, in Experiments 4 to 7, a small amount (10 c.c. N/1 KOH) of alkali was added before distillation. In this case the analysis of the muddy portion of the liquid showed a small amount of the ammonium salt to have been absorbed by the solid, but nothing comparable to the effect required in a sewage filter. Moreover, the slight removal of ammonia demonstrated would appear to be a chemical rather than a physical phenomenon, alkali being necessary to free the absorbed ammonia from the solid.

In none of these experiments, however, was coke itself employed, and the surface of solid was very small in comparison with the amount of liquid taken;

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* In Experiments 6 and 7 (Table IX) the flask was not thus exhausted, and the agreement of their results with those of previous experiments indicates that this precaution is unnecessary.

the following further experiments were therefore made, and confirmed the preceding ones.

*Experiment* 8.—Exactly the same coke as had been used for the filters was taken and thoroughly washed and dried. An amount occupying a volume of 30 c.c. was placed in a flask with 50 c.c. $NH_4Cl$ solution of concentration equal to about 5 parts ammonia per 100,000. In a control flask 50 c.c. of the liquid was placed alone. After 24 hours and after 48 hours the liquids in the two cases were examined, a small quantity (0·5 c.c. to 1 c.c.) being removed, diluted to 50 c.c. with $NH_3$-free water and tested with Nessler's reagent. In no case was the reaction fainter where the liquid had been in contact with the coke. (It was shown that the coke did not of itself yield ammonia by a control experiment in which $NH_3$-free water replaced the $NH_4Cl$ solution.)

*Experiment* 9.—Coke, which had been thoroughly washed, dried, and sterilised, was placed in a cylinder to form a small filter, and a solution of $NH_4Cl$ (10 parts $NH_3$ per 100,000) allowed to drop slowly through.* The filter occupied a volume of about 1 litre and during the first hour 50 c.c. came through, while in 15 hours a total of 500 c.c. was filtered. The first filtrate of 50 c.c. was tested for ammonia and compared with the original liquid. The tint given by the filtrate (after suitable dilution and addition of Nessler's reagent) was, if at all, only a shade paler, indicating only a negligible difference. After a second hour the filtrate was again compared with the control, and a similar result was obtained. The filtrate coming through in the next 13 hours was similarly tested, but no absorption of ammonia was detected.†

It may be objected that experiments with raw, cleansed, filtering material are not applicable to the occurrences in the mature filter, where the surface of the coke is probably coated, in some manner not yet investigated, and might possess the faculty of absorbing ammonia in a manner similar to that already demonstrated in the case of certain colloidal substances.‡ Therefore it is hoped, in the future, to make experiments with matured coke, eliminating, if possible, the action of bacteria. The available evidence is, however, opposed to such absorption, for it is in the uppermost layers of the filter that such a coating would be greatest, and yet disappearance of ammonia, at any rate during the maturing period, has been shown to take place lower down, and in any case coinciding, both as regards time and place, with nitrification.

Upon consideration of the experimental data at present available, one is therefore inclined to reject the current theory of nitrification and to consider the disappearance and oxidation of the ammonia to be parts of one process, which is carried out by the nitrifying bacteria in the time taken by the sewage to pass through the filters.

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* The concentration of ammonia was greater than in the preceding experiments, where it approximated to that in ordinary sewage; here a more concentrated liquid was employed, in order to be comparable with the diluted urine which was then being treated on the filters.

† These last two experiments are in perfect accord with some of A. Mayer ('Lehrbuch d. Agrikulturchem.,' 1871), who showed that pure carbon in a porous condition was unable to effect any significant absorption with many salts long known to be absorbed by the soil.

‡ Van Bemmeden, 'Landw. Versuchsst.,' vol. 35, 1888, 'Zeitschr. f. physikal. Chem., vol. 18.

For these experimental filters the time taken for the passage was measured directly.* For the Vienna continuous filters on July 1, 1901, the time was approximately 3¼ hours for the tall and medium filters and only 5 minutes for the short filter (nitrogen oxidation in this filter had not then progressed beyond formation of nitrites). For the Munich continuous filters on March 17, 1903, the time was 2 hours for the tall filter, and ½ hour for the short one. At this date, a too concentrated sewage was being employed, and the filters were not at their best, but still four-fifths of the ammonia in the sewage was oxidised while passing through the tall filter.

### Section IV.—*General Conclusions.*

1. Nitrification of ammonia during sewage purification occurs in two stages which may be referred to the activity of two classes of bacteria, one producing nitrites, and the second oxidising the nitrites to nitrates. These bacteria exist not only in the substance of the filter, but are also carried away in large quantities in the filtrates.

2. These organisms belong to the same group as those concerned with nitrification in the soil, isolated by Winogradsky. It is, at first, difficult to understand how organisms so susceptible to the presence of organic matter are able to live and do their work in sewage filters. The following, one or all, form possible explanations.

(*a*) The nitrifying bacteria may be, to a certain extent, protected by the presence of other organisms, and this view is strengthened by the results of certain experiments with the nitrate-producer, in symbiosis with such organisms, made in the course of the present investigation.

(*b*) It has been shown that porous materials, such as coke, are able to retain upon their surface complicated organic substances of high molecular weight, when these are presented in solution. We may suppose this absorption (together with the mechanical separation of the suspended materials in sewage, also largely of organic origin) to take place principally in the upper layers of the filter. The nitrifying organisms will then be able to live and multiply lower down in the filter where the amount of organic matter present will be comparatively small, and this view has been experimentally confirmed in the present work.

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* 100 c.c. of a 2-per-cent. solution of sodium chloride were sprinkled over the top of the filter (that being the volume of liquid usually delivered at each discharge of the siphon). The filtrates were then continuously tested with silver nitrate until a copious precipitation was obtained; the ordinary sewage filtrate yielded only a slight reaction with silver nitrate. The passage of liquids through such filters is a very complicated process and one not yet thoroughly investigated, and though, doubtless, the times thus determined may be considered to apply to the *majority* of the liquid going on at any particular time, they must still, strictly speaking, be regarded as approximate and indeed minimal values.

(*c*) It has been lately shown by *Wimmer*,* in the case of the nitrate organism, that a porous medium has a markedly mitigating effect when organic matter is present, and the coke and other materials of which sewage filters are made, are selected mainly on account of their porosity. It is only fair, however, to state that Wimmer's experiments were not made with absolutely pure cultures, and part of the beneficial effect observed may have been due to a symbiosis, though, from the nature of his experiments, it would seem unlikely.

(*d*) The nitrifying bacteria are doubtless present in very great numbers in the filters, and this may assist them in withstanding the effect of organic matter. This view is based upon certain observations of *Winogradsky* and *Omeliansky*,* in which nitrifying organisms, if present in sufficient quantity, were shown to withstand amounts of organic matter otherwise inhibiting them.

3. In the maturing of sewage filters, the two stages of nitrification may be markedly separate in time (Vienna experiments), or may be both developed together (Munich experiments). This difference is correlated with the greater or less ammoniacal content of the sewage. In the stronger sewage used for the Vienna filters, the well known inhibitory action of abundance of ammoniacal compounds (especially of free ammonia and carbonate of ammonia,† which are so largely represented in the sewage), presumably retarded the development of the nitrate-producer, until the nitrite-producer was sufficiently well established to be converting most of the ammonia into nitrites.

4. As a result of special experiments with coke, and of analyses of the filtrates at different depths of the filters, and at different stages during the maturing period, it would appear that there is no evidence of absorption of free and saline ammonia without contemporaneous nitrification. Further research is necessary, but the theory of a previous physical "adsorption" of ammonia and subsequent slower nitrification would appear, at present, to be without experimental foundation.

5. One is therefore inclined, in the present state of our knowledge, to consider the process of nitrification, during the filtration of sewage through

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* See footnote, p. 242.

† Löhnis, 'Centralbl. f. Bakt.,' 2 Abt., 13, 1904, and Boulanger and Massol, 'Comptes Rendus,' vol. 140, 1905.

such filters, to be an extremely rapid biological process, requiring for its completion only the time taken for the liquid to pass through the filter (approximately 2 to 3 hours, possibly a little more). The rapidity of the process is probably to be explained by the very great number of nitrifying bacteria present and the very efficient aëration which obtains. In such filters also, the general conditions are ideal for quick action, as the continuous trickle secures rapidity of diffusion, and forms a great contrast to the much slower effect in stationary fluids.

6. Temperature has a marked influence upon the oxidation of sewage, a higher temperature being noticeably more favourable. This indicates that the efficiency of sewage filters in practice would be much increased if at a reasonable cost they could be artificially maintained at a warm temperature during the winter.*

7. The previous conclusions are chiefly drawn from experiments with continuous filters, but filters working as contact beds were also investigated and the two methods compared. On the "ammonia adsorption theory," the contact method should have proved the most efficient. This, however, was not found to be the case. The advantages of the continuous method would seem to lie in the much more complete aëration and efficient diffusion, and also in the stratified distribution in the filter of the different stages of the sewage purification. Some of the present experiments were quite comparable with practical installations as regards quantity of liquid treated, concentration of nitrogen, etc., and the results were much more satisfactory than those usually obtained in practice. The obvious difficulty in practical employment of continuous filters is with regard to the solids in suspension, which can only be permitted upon the filter to a small extent without risk of clogging. The present experiments were all made with roughly filtered solutions, but the difficulty could be met in practice by a previous screening of the sewage or by passing it through a septic tank. Should clogging occur, it will probably take place in the superficial layers and could be remedied by simple mechanical treatment. In the case of contact beds, however, clogging necessitates the cleansing of the whole bed, an exceedingly costly process. From these considerations, and as a result of the present experimental study, the method of continuous filtration would appear to be a most advantageous method of purifying sewage.

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* Ducat filters, which are artificially warmed in cold weather, perform an amount of nitrification which is well above the average.

## Tables I—IX.

In these tables the estimations of "free and saline ammonia" are expressed as *ammoniacal nitrogen*, those of "albuminoid ammonia" as *albuminoid nitrogen*, those of nitrites as *nitrous nitrogen*, and those of nitrates as *nitric nitrogen*, all in parts of nitrogen per 100,000 by weight.

Nitrites were estimated by the metaphenylenediamine colorimetric method unless otherwise stated, the sum of nitrites and nitrates by the indigo method (Vienna analyses), or by the copper-zinc couple method (Munich analyses).

Throughout these tables the grades of reactions are represented as follows: reaction absent by 0, a trace, or faint reaction, by *f*, definite reaction by +, and intense reaction by ++.

*Oxidisability* is expressed in parts oxygen absorbed per 100,000 by weight, less that absorbed by any nitrites present. In figures marked with an asterisk the oxidisability was approximately gauged by the difference between the oxygen absorbed in the cold and on boiling.

Table IA.—Vienna Continuous Filters, showing the Progress of Nitrogen Oxidation during Maturing. Filters all started on February 20, 1901. Figures in brackets are weeks elapsed since the start.

| Course of oxidation. | Tall filter. | Medium filter. | Short filter. |
|---|---|---|---|
| Oxidised N. first detected in filtrate | March 18<br>(4 w.) | March 21<br>(4 w.) | March 28<br>(5 w.) |
| Nitrites present in quantity, no nitrates | March 28*<br>(5 w.) | March 28†<br>(5 w.) | July 1‡<br>(19 w.) |
| Nitrates also present in quantity | May 24<br>(11 w.) | May 13<br>(9½ w.) | |
| Nitrates alone present ............ | July 1<br>(19 w.) | July 1<br>(19 w.) | |

* 10 parts per 100,000 N. as $N_2O_3$. † 1·3 parts ditto.

‡ 4·1 parts ditto, nitrates also present in small amount.

Table IB.—Munich Experiments. Filters all started April 1, 1903.

| Course of oxidation. | Continuous filters. | | | Contact filters. | | |
|---|---|---|---|---|---|---|
| | Tall. | Medium. | Short. | Tall. | Medium. | Short. |
| Oxidised N. first detected in filtrate | May 2<br>(4 w.) | May 2<br>(4 w.) | May 2<br>(4 w.) | May 16<br>(6½ w.) | May 16<br>(6½ w.) | May 16<br>(6½ w.) |
| Nitrites present in quantity, nitrates present | May 18<br>(7 w.) | May 23<br>(7½ w.) | | | | |
| Majority of oxidised N. present as nitrates | May 27<br>(8 w.) | — | — | June 25<br>(12 w.) | June 25<br>(12 w.) | June 25<br>(12 w.) |
| Nitrates alone present... | June 8<br>(10 w.) | June 13<br>(10½ w.) | June 13<br>(10½ w.) | July 9<br>(14½ w.) | July 9<br>(14½ w.) | July 9<br>(14½ w.) |

Table II.—Analyses of Sewage Affluent and Filtrates. Analyses 1—23 deal with the Vienna continuous filters, all started 20.2.01; Analyses 24—36 deal with the Munich continuous filters, all started 1.4.03. The results are expressed in parts per 100,000.

| No. of Analysis. | Date. | Material analysed. Height of Filter. | Ammoniacal nitrogen. | Albuminoid nitrogen. | Nitrous nitrogen. | Nitric nitrogen. | Total nitrogen, Kjeldahl. | Total nitrogen calculated. | Oxidisability, in terms of O. |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 7.3.01 | Raw sewage ............ | Equal to sewage. | — | 0 | 0 | — | — | 10·3 |
| 2 | 7.3.01 | Filtrate—tall ......... |  | — | 0 | 0 | — | — | 5·9 |
| 3 | 7.3.01 | ,, —medium ... |  | — | 0 | 0 | — | — | 5·4 |
| 4 | 7.3.01 | ,, —short......... |  | — | 0 | 0 | — | — | 6·4 |
| 5 | 13.3.01 | Raw sewage ............ | — | — | 0 | 0 | — | — | 15·3 |
| 6 | 13.3.01 | Filtrate—tall ......... | — | — | 0 | 0 | — | — | 4·2 |
| 7 | 13.3.01 | ,, —short......... | — | — | 0 | 0 | — | — | 6·6 |
| 8 | 18.3.01 | Raw sewage ............ | — | — | 0 | 0 | — | — | 15·3 |
| 9 | 18.3.01 | Filtrate—short......... | — | — | 0 | 0 | — | — | 5·4 |
| 10 | 28.3.01 | Raw sewage ............ | 15·2 | 2·0 | 0 | 0 | 17·2 | 17·2 | 42·8 |
| 11 | 28.3.01 | Filtrate—tall ......... | 1·2 | 0·38 | 10·1 | 0 | — | 11·6 | 5·1 |
| 12 | 28.3.01 | ,, —medium ... | 15·1 | — | 1·6 | 0 | — | — | — |
| 13 | 28.3.01 | ,, —short......... | 15·4 | — | *f* | — | — | — | 4·6 |
| 14 | 1.4.01 | Raw sewage ............ | 14·7 | 2·0 | 0 | 0 | 17·2 | 16·7 | — |
| 15 | 1.4.01 | Filtrate—tall ......... | 3·8 | 2·3 | 10·4 | 0 | — | 16·5 | — |
| 16 | 13–15.5.01 | Raw sewage ............ | 14·7 | 2·5 | 0 | 0 | — | 17·2 | 13·5 |
| 17 |  | Filtrate—medium ... | 0·3 | 0·4 | 5·7 | 3·1 | — | 9·5 | 4·1* |
| 18 |  | ,, —short......... | 10·8 | 1·3 | *f* | — | — | 12·1 | 5·2 |
| 19 | 24.5.01 | ,, —tall ......... | 0·3 | — | 6·0 | 1·1 | — | — | — |
| 20 | 1.7.01 | Raw sewage ............ | 11·9 | — | 0 | 0 | — | — | — |
| 21 | 1.7.01 | Filtrate—tall ......... | 0·1 | — | 0 | 7·8 | — | — | — |
| 22 | 1.7.01 | ,, —medium ... | 4·1 | — | *f* | 5·0 | — | — | — |
| 23 | 2.7.01 | ,, —short......... | 3·7 | — | 4·1 | *f* | — | — | — |
| 24 | 19.5.03 | Raw sewage ............ | 3·5 | 1·6 | 0 | 0 | — | 5·1 | 11·6 |
| 25 | 19.5.03 | Filtrate—tall ......... | 0·2 | 0·2 | 2·5 | 0·4 | — | 3·3 | 2·4 |
| 26 | 23.5.03 | Raw sewage ............ | 3·6 | 1·4 | 0 | 0 | — | 5·0 | — |
| 27 | 23.5.03 | Filtrate—medium ... | 0·1 | 0·2 | 1·2 | 1·5 | — | 3·0 | — |
| 28 | 26–27.5.03 | Raw sewage ............ | 4·0 | 1·6 | 0 | 0 | — | 5·6 | 14·3 |
| 29 |  | Filtrate—tall ......... | 0·07 | 0·2 | 0·6 | 2·0 | — | 2·9 | — |
| 30 | 15–17.6.03 | Raw sewage ............ | 2·0 | 1·6 | 0 | 0 | — | 3·6 | 10·9 |
| 31 |  | Filtrate—tall ......... | 0·04 | 0·2 | 0 | 2·1 | — | 2·3 | 3·6 |
| 32 |  | ,, —medium ... | 0·07 | 0·24 | 0 | 1·7 | — | 2·0 | 4·1 |
| 33 | 24.6.03 | Cows' urine, 1 p. c. ... | 13·2 | 3·8 | 0 | 0 | — | 17·0 | 10·1 |
| 34 | 24.6.03 | Filtrate—tall ......... | 0·08 | 0·15 | 0 | 8·3 | — | 8·5 | 1·61 |
| 35 | 13.7.03 | Cows' urine, 1 p. c. ... | 10·0 | 4·1 | 0 | 0 | — | 14·1 | — |
| 36 | 13.7.03 | Filtrate—tall ......... | 0·05 | 0·17 | 0 | 8·1 | — | 8·3 | — |

Table III.—Showing the Course of Oxidation at Successive Depths in Tall and Medium Filters (Vienna).

| No. | Date. | Constituents. | Sewage. | Filtrate at successive depths. | | | |
|---|---|---|---|---|---|---|---|
| | | | | Tap No. 1, 50 cm. | Tap No. 2, 100 cm. | Tap No. 3, 150 cm. | Tap No. 4, 200 cm. |
| | | Tall filter. | | | | | |
| 1 | 24.5.01 | Ammoniacal N. | 12·78 | 11·91 | 2·27 | 0·39 | 0·29 |
| 2 | 24.5.01 | Oxidised N....... | 0 | *f* | + | + + | + + † |
| 3 | 27.5.01 | Oxidisability* ... | 9·2* | 3·4* | 2·6* | 3·0* | 2·3* |
| 4 | 27.5.01 | Nitrites........... | — | *f* | *f* | No. 2 × 5 | No. 2 × 7 |
| 5 | 1.7.01 | Ammoniacal N. | 11·9 | 2·5 | 0·2 | 0·04 | 0·06 |
| 6 | 1.7.01 | Nitrites........... | 0 | *f* | 0 | 0 | 0 |
| 7 | 1.7.01 | Nitrates ........ | 0 | + | + | + | 7·8 |
| | | Medium filter. | | | | | |
| 8 | 1.7.01 | Ammoniacal N. | 11·9 | 8·1 | 4·12 | | |
| 9 | 1.7.01 | Nitrites........... | 0 | *f* | *f* | | |
| 10 | 1.7.01 | Nitrates ........ | 0 | *f* | 4·96 | | |

In 2 the test was made with diphenylamine, in 3 the estimations are approximate.

The high numbers in filtrate of 8 are due to deterioration in efficiency of the filter following partial clogging.

† Nitrous N = 6·0, nitric N = 1·1.

Table IV.—Munich Continuous Filters from June 24, 1903, to November 27, 1903, using Cows' Urine diluted 1 in 100; this contained 14—17 parts $NH_3$ per 100,000 and no oxidised nitrogen. In cases marked with an asterisk the urine was only diluted to 1 in 50.

| Date. | Tall filter. | | | Medium filter. | | | Short filter. | | |
|---|---|---|---|---|---|---|---|---|---|
| | $NH_3$ per 100,000. | Nitrite re-action. | Nitrate re-action. | $NH_3$ per 100,000. | Nitrite re-action. | Nitrate re-action. | $NH_3$ per 100,000. | Nitrite re-action. | Nitrate re-action. |
| 26.6.03 | *f* | 0 | + + | *f* | 0 | + + | 1 | *f* | + + |
| 30.6.03 | 0·1 | 0 | + + | 0·1 | 0 | + + | 2—3 | *f* | + + |
| 9.7.03 | 0·05 | 0 | + + | 2—3* | + | + + | 1—2 | + | + + |
| 13.7.03<br>15.7.03 | 0·2 | 0 | + + | 6—7* | *f* | + + | 2—3 | *f* | + + |
| 23.7.03 | 0·05 | 0 | + + | 4—5* | *f* | + + | 1—2 | + | + + |
| 24.10.03 | 0·05 | 0 | + + | 0·05 | 0 | + + | 0·1 | 0 | + + |
| 27.11.03 | 0·02 | 0 | + + | 0·04 | 0 | + + | 0·04 | 0 | + + |
| 20.1.04 | 5·0* | *f* | + + | 8—10* | + + | + + | 15* | + | + + |

The ammonia estimations by direct Nesslerisation are only approximate; nitrites were tested for by acidified starch-zinc-iodide; nitrates by diphenylamine (only specific if nitrites are absent or inconsiderable).

Table V.—Enumeration of Organisms in Filtrates.

| Date. | Munich continuous filters. | No. of organisms per cubic centimetre. | | | Time subcultures were kept. |
|---|---|---|---|---|---|
| | | Growing in bouillon. | Nitrite-producer. | Nitrate-producer. | |
| 6.6.03 | Tall | 10,000 | 10,000 | 100* | 6 weeks |
| 6.6.03 | Medium | 1,000 | 10,000 | 100 | 6 " |
| 6.6.03 | Short | 100,000 | 10,000 | 100 | 6 " |
| 12.6.03 | Tall | 10,000 | 10,000 | 10,000 | 4 months |
| 12.6.03 | Medium | 100,000 | 100,000 | 1,000 | 4 " |
| 12.6.03 | Short | 100,000 | 10,000 | 100 | 4 " |
| 18.6.03 | Tall | 10,000 | 10,000 | 100 | Former 4 m. |
| 18.6.03 | Short | 1,000 | 1,000 | 10 | Latter 6 w. |
| 24.6.03 | Tall | 10,000 | 1,000 | 10,000 | 4 months |
| 24.6.03 | Medium | 10,000 | 1,000,000 | 10,000 | 4 " |
| 27.6.03 | Tall | 1,000 | — | 10,000 | 4 " |
| 27.6.03 | Medium | 10,000 | — | 10,000 | 4 " |

* Culture containing 0·01 c.c. filtrate, used for dilution experiment on p. 252.

Table VI.—Results of Inoculating two different Impure Nitrite-producer Cultures, "*a*" and "*b*," into Ammoniacal Media, both directly and after passing through one or two generations in bouillon. + + indicates an intense nitrite reaction.

| Series. | Sequence of inoculation. | "*a*," three weeks old. | "*a*," twelve days old. | | "*b*," three weeks old. | | "*b*," four weeks old. |
|---|---|---|---|---|---|---|---|
| 1 | From original material | + + | — | — | + + | + + | + + |
| 4 | From Series 1 | — | + + | + + | + + | + + | — |
| 3 | From original through bouillon (Series 2) | 0 | 0 | 0 | + + | + + | + + |
| 6 | From Series 2 again, through bouillon (Series 5) | — | — | — | 0 | 0 | — |

Table VII.—Results of Inoculating into Nitrite Medium of Five Strains of a "Symbiotic" Culture of Nitrate-Producer, "*d*," after Successive Generations in Bouillon. + indicates a positive, + + an intense reaction.‡

| Dates of successive inoculation into bouillon. | Date of inoculation from bouillon into nitrite medium. | Date of testing preceding cultures. | "*d* 1" | | "*d* 2" | | "*d* 3" | | "*d* 4" | | "*d* 5" | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Nitrites. | Nitrates. | Nitrites. | Nitrates. | Nitrites. | Nitrates. | Nitrites. | Nitrates. | Nitrites. | Nitrates. |
| 17.12.03 → | 23.12.03 | 12.1.04 | 0 | + + | 0 | + + | 0 | + + | | | | |
| ↓ 12.1.04 → | 18.1.04 | 3.2.04 | + + | 0 | 0 | + + | 0 | + + | 0 | + + | 0 | + + |
| ↓ 18.1.04 → | 26.1.04 | 10.3.04 | + + | 0 | 0 | + + | 0 | + + | 0 | + + | + + | 0 |
| ↓ 26.1.04 → | 3.2.04 | 10.3.04 | + + | 0 | + + | 0 | + | + | 0 | + + | + + | 0 |

Table VIII.—Showing the Different Behaviour of Pure and of "Symbiotic" Bouillon-Cultures of the Nitrate-Producer when Inoculated into Nitrite Medium, the latter tested for nitrites and nitrates 31 days after inoculation; + indicates a definite, + + an intense reaction.‡

| Reactions of the nitrite medium. | Pure cultures, bouillon clear. | | | | | | "Symbiotic" cultures, bouillon showing growth. | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | No. 25. | | No. 39. | | No. 67. | | No. 49. | No. 29. | | No. 12. | No. 21. | |
| Nitrite ...... | + + | + + | + + | + + | + + | + + | 0 | 0 | 0 | 0 | + | + |
| Nitrate ...... | 0 | 0 | 0 | 0 | 0 | 0 | + + | + + | + + | + + | + | + |

Cultures No. 49 and No. 29 were strains of culture "*d*"; cultures No. 12 and No. 21 were of different origin.

‡ With cultures still containing nitrites, evaporation with ammonium chloride preceded the diphenylamine test for nitrates.

Table IX.—Experiments upon the Absorption of Ammonium Chloride from Dilute Solution by Various Finely Divided Solids.

| No. | Solid tested; weight taken. | Original solution of $NH_4Cl$ added. | | Liquid after standing, ammonia content per 100,000 parts. | | Ammonia in blank control in thousandths of a milligramme. | |
|---|---|---|---|---|---|---|---|
| | | Volume of liquid in c.c. | Ammonia content per 100,000 parts. | Upper clear liquid. | Lower turbid liquid. | Clear liquid. | Turbid liquid. |
| 1 | $BaSO_4$, 1 gramme ... | 100 | 1·00 | 1·02 | 0·91 | 0 | 0 |
| 2 | Sand, 2 grammes ...... | 100 | 5·00 | 4·30 | 4·04 | 2 | 0 |
| 3 | Sand, 2 „ ...... | 50 | 5·00 | 4·14 | 3·78 | 3 | 0 |
| 4 | Clinker, 2 grammes ... | 50 | 1·00 | 0·82 | 0·89 | 2 | 0 |
| 5 | Sand, 2 grammes ...... | 50 | 1·00 | 0·75 | 1·03 | 5 | 0 |
| 6 | Sand, 2 „ ...... | 50 | 1·00 | 0·83 | 1·05 | 3 | 0 |
| 7 | Sand .................... | — | 1·00 | 0·85 | 0·99 | | |

In Experiments 4—7, 10 c.c. N/1 KHO was added before distilling off the ammonia.

# *The Action of Anæsthetics on Living Tissues.* Part I.—*The Action on Isolated Nerve.*

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## *Introduction.*

The action of anæsthetics on isolated nerve has already been studied by Waller* as regards the effect on the negative variation in the sciatic of the frog, and by myself in mammalian nerves.† In the course of these researches it became evident that the anæsthetics used (chloroform, ether, carbon dioxide) affected not only the negative variation, but also the injury current, and as this action has not been studied before, as far as I am aware, it seemed desirable to investigate the matter not only in nerve, but also in other tissues.

The inquiry falls naturally under two heads: first, in how far the phenomena throw light on the processes of nerve action; and, secondly, as regards the chemical and physical action of anæsthetics on the animal protoplasm generally. These are obviously only parts of the same story, but for convenience I have considered the subject mainly under the first heading in this part, leaving the more general question to a future occasion.

## *Experiments.*

If the sciatic nerve of a frog be taken and a fresh transverse section be made at the distal end, and this end be placed in contact with one non-polarisable electrode, while another electrode rests on an uninjured longitudinal surface, the galvanometer will indicate a difference of potential which declines at a certain rate. This can be measured either by photographing the movement of the galvanometer spot, or, more accurately, by balancing against a known potential, reading the potentiometer at convenient intervals of time, and plotting the figures on squared paper; this latter method eliminates any change in the resistance of the object. The curve obtained is concave to the abscissæ, neglecting a variation which is occasionally found during the first five minutes of the experiment.

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* Waller, "Lectures on Animal Electricity," 1897, and 'Proc. Physiol. Soc.,' November 13, 1897, etc.

† 'Roy. Soc. Proc.,' vol. 71, p. 264, and vol. 73, p. 166.

If, when this curve has assumed a typical form, $CHCl_3$ vapour be applied to the nerve, a sudden drop is observed. If the vapour is weak, this is followed by a recovery when the $CHCl_3$ is removed, if the vapour is strong (12 per cent. or over) the drop is permanent (fig. 1). This may be due

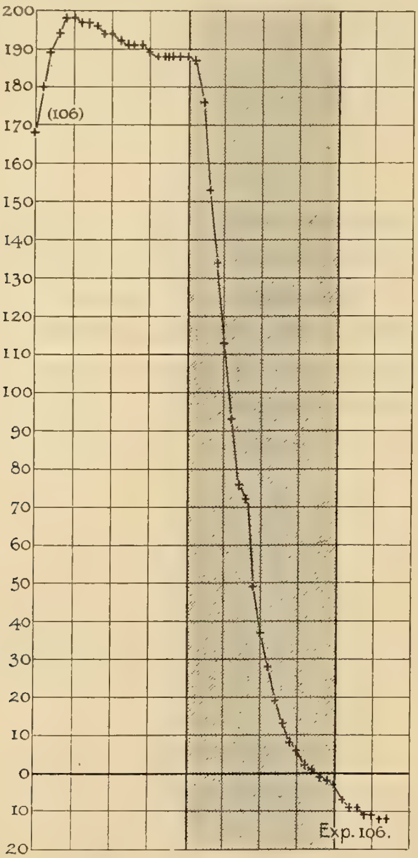

Fig. 1.—Sciatic of Frog. $CHCl_3$ vapour at 12·5 per cent. between the vertical bars. Ordinates 1 mm. = 0·0001 volt. Abscissæ 1 mm. = 15 seconds.

to events occurring either at the cut end, or at the longitudinal surface or both, the experiments in Series II were undertaken to determine which of these factors predominated.

### *Series II.*

The nerve-chamber was divided by a transverse gas-tight partition of modelling wax, so that the anæsthetic could be applied either to the cut end A or to the longitudinal surface B (fig. 2). The figures obtained from the potentiometer readings have been plotted out to a scale on the ordinate of which one division = 0·0001 volt, and on the abscissa one division = 15 seconds. The point at which the anæsthetic was applied was taken as the zero, and the relative heights plotted from this, the values are, therefore, relative and not absolute (fig. 3). The figures under the middle part of each curve indicate the percentage of $CHCl_3$ vapour, as measured by Waller's densimetric method.* The vapour was contained in a bag of gold-beaters' skin; the leakage from this was ascertained to be small during the time taken by an experiment of this kind. Here, as elsewhere in this paper, I have only considered experiments where the results are sufficiently concordant to admit of a consecutive series being employed without having to reject isolated experiments. Any unsuspected errors are, therefore, of a constant magnitude throughout a given series.

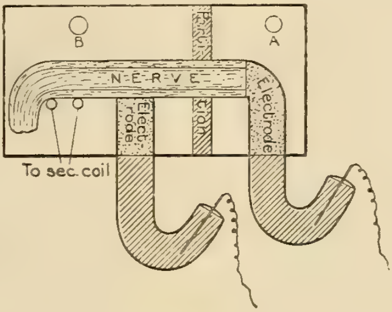

Fig. 2.—Nerve chamber.

It will be seen that—

(1) $CHCl_3$ to cut end of nerve causes an *increase* in the injury current.

(2) This increase is roughly proportional to the strength of the $CHCl_3$ vapour up to about 12 per cent., greater concentration then gives no further increase.

(3) $CHCl_3$ to longitudinal surface causes a decrease of the injury current.

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* Waller and Geets, 'British Medical Journal,' June 20, 1903.

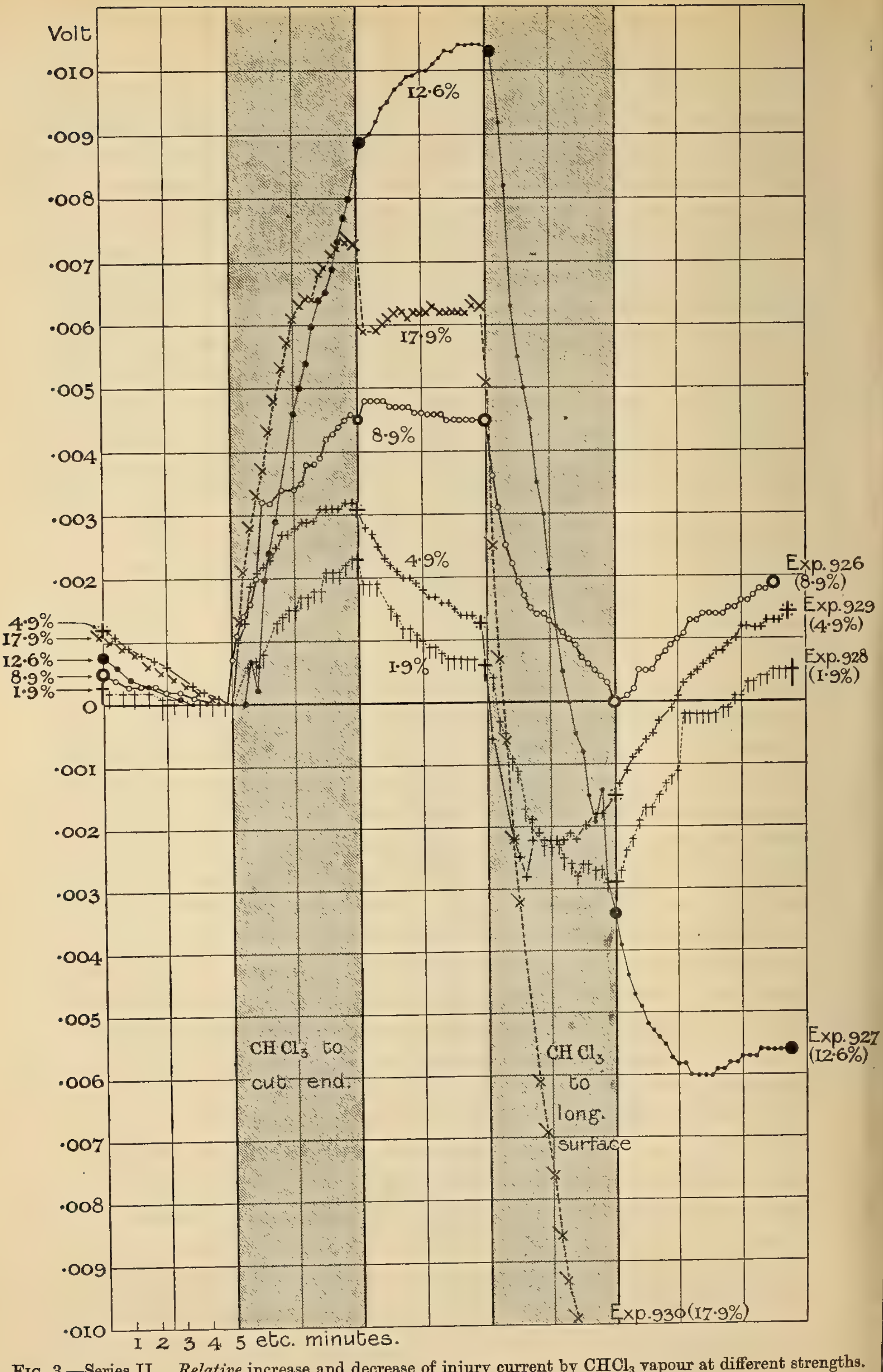

FIG. 3.—Series II. *Relative* increase and decrease of injury current by $CHCl_3$ vapour at different strengths. Ordinates 1 mm. = 0·0001 volt. Abscissæ 1 mm. = 15 seconds.

(4) Recovery takes place when the vapour is weak (9 per cent. or less), not occurring when the strength is 12 per cent. or more.

### *Series III.*

The method adopted in Series II, while suitable for percentages of $CHCl_3$ from 1 to 10, is defective in many ways, and an apparatus was designed to deliver a constant high percentage of $CHCl_3$, saturated with water vapour, and at a constant temperature. This consisted of a foot bellows with a branched delivery tube, one limb of which passes to seven Fresenius flasks, the first three containing $CHCl_3$, the next water, the next two contain a mixture of $CHCl_3$ and water, and the last is empty. The apparatus delivers moist $CHCl_3$ vapour at a concentration of 13 to 17 per cent. (depending on the temperature of the first flask) with a variation in strength of about 0·5 per cent. and a temperature variation of about 0·4° C., and by estimating the strength of the vapour after it has passed through the nerve-chamber, and noting the temperature before and after each experiment, these residual errors can be allowed for. The five flasks on the other limb of the air supply contain water, and a small stream of air passing through them serves to ventilate the other part of the nerve-chamber, thus guarding against accidental escape of $CHCl_3$. A similar apparatus is used for ether: in this case the percentage is about 45. Unless otherwise stated, all further experiments were made with this apparatus, with occasional modifications.

In this series the strength of the $CHCl_3$ vapour was 12·5 ($\pm$0·5 per cent.), and was applied for 10 minutes. The details of the various experiments differed. The values are now absolute, and have been corrected for electrode current. Each ordinate division = 0·002 volt; each abscissa division = 15 seconds.

In Experiments 108 and 107 (nerves of Frog A) the $CHCl_3$ was applied first to the longitudinal surface and then to the cut end. In Experiment 109 (Frog B) the same course was followed, only without making a fresh section, the curve therefore begins close to the zero line. In these three experiments the curves fall at first, the effects being naturally opposite in sign to those in the next three experiments—Nos. 111, 110, and 117—where the cut end is first affected. No. 110 is practically a repetition of Series I, No. 111 the same without a fresh section, and No. 117 shows the effect of ether applied for five minutes instead of ten.

Considering the first half of each experiment—

(1) $CHCl_3$ (Experiment 111) and ether (Experiment 117) to the distal end of a nerve cause a rise in potential, approximately equal in amount.

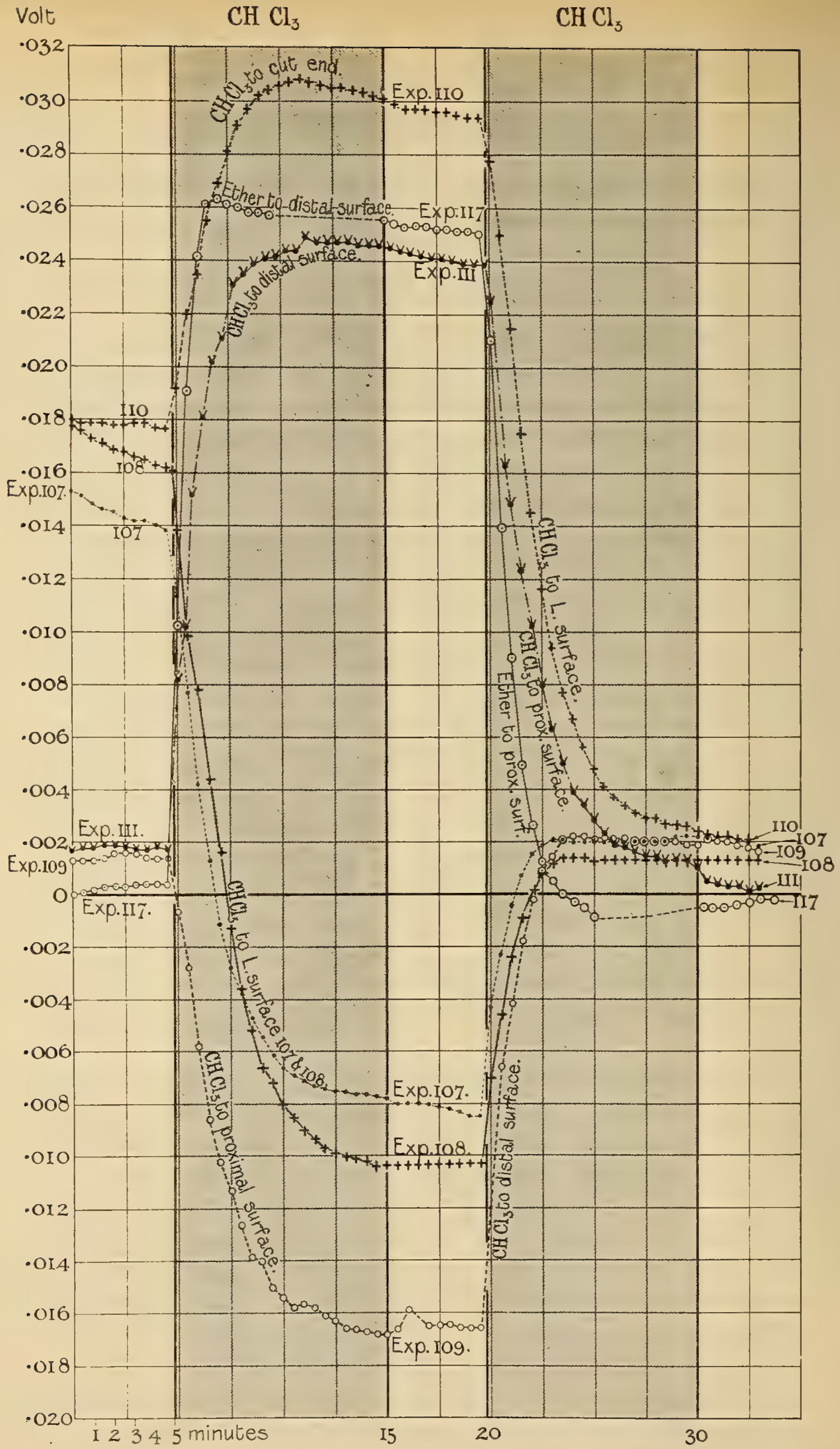

FIG. 4.—Series III. CHCl$_3$ of 12·5 per cent. and ether of 50 per cent. to either end of the nerve alternately. Ordinates 1 mm. = 0·0002 volt, abscissæ 1 mm. = 15 seconds. Expts. 107 and 108, CHCl$_3$ to L. surface and then cut end. Expt. 110, cut end and then L. surface. Expts. 109 and 111, CHCl$_3$ to different ends *without* a previous section. Expt. 117, ethers 50 per cent. to cut end and then L. surface.

(2) The effect of a previous section (110) is to produce a somewhat larger rise.

(3) $CHCl_3$ to the proximal end produces a fall in potential (107, 108, 109). When there is no previous section (109) the experiment is obviously identical with Experiment 111; when there is a previous section, the total effect is less. In other words, if

$x$ = effect of section, and
$y$ = effect of $CHCl_3$ in the first part of each experiment,

$$\begin{aligned} \text{Exps. 109 and 111} &= y, \\ \text{,, } \quad 107 \text{ ,, } \quad 108 &= x-y, \\ \text{,, } \quad 110 \qquad\quad &= x+y. \end{aligned}$$

These results are discussed subsequently in connection with the question as to whether $x$ and $y$ are due to the same mechanism in the nerve or not. Later experiments (Series V and VI) point to an affirmative answer; as regards this present series, it appears that $y$ is of the same order of magnitude as $x$, whether $CHCl_3$ or ether is used, and that $y$ has the same direction of current as $x$.

A few experiments were made with alcohol vapour; this causes an effect of the same character as $CHCl_3$ and ether, but as it also causes an enormous increase in the resistance of the tissue, due to drying, the experiments were not proceeded with further.

When, in the second part of each experiment, the anæsthetic is applied to the other part of the nerve, the final result is similar to that of Series I, where the whole nerve is at once anæsthetised. As can be seen, there is a small residual E.M.F., occasionally (in other experiments not quoted here); this may reach a value of 0·005 to 0·006 volt, the exact significance of this is still uncertain.

### *Series IV.*

In this series, $CHCl_3$ and ether vapours of different strength were applied simultaneously to both sides of the nerve. The result could have been predicted from an examination of Series I and II, but is a useful control, as certain sources of error are eliminated.

| Experiment. | Central End. | Peripheral End. | E.M.F. initial. | E.M.F. final. |
|---|---|---|---|---|
| | per cent. | per cent. | | |
| 931 | 51·0 (ether) | 16·1 ($CHCl_3$) | +33 | − 1 |
| 932 | 16·1 ($CHCl_3$) | — | − 8 | −233 |
| 933 | 12·5 ($CHCl_3$) | 16·5 ($CHCl_3$) | +13 | − 18 |
| 934 *a* | 8·0 ($CHCl_3$) | 14·5 ($CHCl_3$) | −16 | −184 (*a*) |
| 934 *b* | 14·5 ($CHCl_3$) | | | − 16 (*b*) |
| 113 | 54–32·5 (ether) | 12·3 ($CHCl_3$) | −25 | − 7 |

If the small final values under 20 be disregarded, these experiments show that:—

(1) Ether vapour of 51 to 54 per cent. and $CHCl_3$ of 16 to 12·3 per cent. are identical in their final effects.

(2) $CHCl_3$ of 8 per cent. has a less effect. $CHCl_3$ vapour of about 12 per cent. has, therefore, a maximum action.

(3) Ether acts more quickly than $CHCl_3$ (fig. 5).

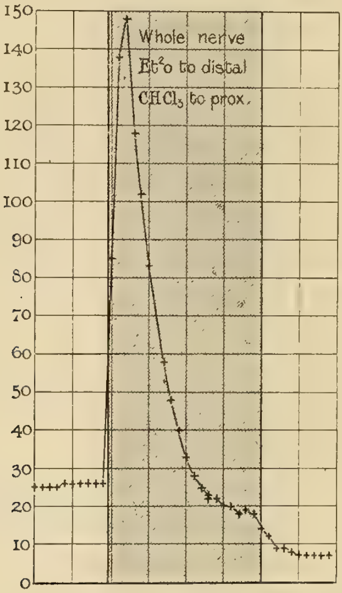

Fig. 5.—Series IV.

### *Control Experiments.*

In order to exclude any possible source of current from electrodes or any unexpected physical cause, control experiments were made with threads moistened with (*a*) salt solution, (*b*) egg albumen, (*c*) egg yolk, and (*d*) fresh blood. These were treated in an exactly similar manner to the nerves in Series II. No electromotive phenomena followed the application of $CHCl_3$ vapour. So also nerves kept in salt solution until they gave no injury current showed no results with anæsthetic vapours. HCl and $NH_4OH$ gave electromotive effects when applied either to threads or nerves, HCl with the same sign as $CHCl_3$, $NH_4OH$ with the reverse. The similarity is therefore only apparent and probably due to diffusion into the electrodes.

### *Series V.*

The effect on the electrical resistance of the application of anæsthetic vapours is of great importance in endeavouring to give an explanation of the facts recorded above, and so, although the question has already been studied by Waller,* I have performed a considerable number of experiments in order to eliminate as far as possible the numerous sources of error present.

The method finally adopted was to place the nerve on non-polarisable electrodes in a chamber, pass in air saturated with water vapour, and take several successive readings until either they remained constant or else the rate of change was determined. Moist $CHCl_3$ vapour was then admitted for five minutes, a reading taken, the chamber washed out with moist air for four minutes, and the final value observed. A linen thread wet with M/10 NaCl solution rested on non-polarisable electrodes in the same chamber, and readings of its resistance were taken alternately with the nerve. A thermometer as close to the nerve and thread as possible gave an approximation to the temperature; as will be seen, the mean variation of this is about 0·4° C., and in view of the difficulty of securing an equal amount of moisture and an equality of salt content in the electrodes, threads and nerves, the temperature error may for the present purpose be disregarded.

Two methods of determining the resistance were used :—

(1) The ordinary Wheatstone bridge, using non-polarisable electrodes and a constant current from one Leclanché cell.

(2) A slightly modified Kohlrausch apparatus, using the same electrodes and alternate currents.

With the control threads the results were identical; both sets of readings show a diminution of resistance of about 2 per cent. after $CHCl_3$, in part due to temperature alterations, in part to other causes. The nerves on the other hand gave markedly different figures with the two methods.

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* Waller, 'Proc. Physiol. Soc.,' November 12, 1898

## Series V.

### A.—Frog's Sciatic. Wheatstone Bridge.

| Experiment. | Resistance before $CHCl_3$ (ohms) $R_1$. | T.° before. | Resistance during $R_2CHCl_3$. | T.° during. | Resistance after $CHCl_3R_3$. | T.° | Percentage of $CHCl_3$ vapour. | $R_3/R_1$. | Notes. |
|---|---|---|---|---|---|---|---|---|---|
| 168 A | 107,300 | 16·8 | 107,800 | 17·2 | 106,200 | 16·2 | 14·5 | 0·990 | Thread |
| B | 142,100 | | 150,700 | | 121,700 | | | 0·858 | Nerve |
| 169 A | 92,000 | 16·9 | 94,100 | 17·3 | 93,600 | 17·0 | 15·5 | 1·02 | Thread |
| B | 95,400 | | 102,300 | | 86,700 | | | 0·909 | Nerve |
| 170 A | 110,000 | 16·7 | 108,000 | 17·1 | 105,600 | 17·0 | 17·0 | 0·960 | Thread |
| B | 117,700 | | 107,400 | | 90,700 | | | 0·770 | Nerve |
| 171 A | 115,300 | 16·3 | 114,800 | 16·5 | 111,500 | 16·6 | — | 0·967 | Thread |
| B | 130,400 | | 140,300 | | 114,700 | | | 0·880 | Nerve |
| Mean of 4 thread Experiments (A) | 106,100 | 16·68 | 106,200 | 17·03 | 104,300 | 16·7 | — | 0·984 | |
| Mean of 4 nerve Experiments (B) | 121,400 | | 125,200 | | 103,500 | | | 0·852 | |

Series V—*continued.*

B.—Frog's Sciatic. Kohlrausch

| Experiment. | Resistance before $CHCl_3$ (ohms). | T.° before. | Resistance during $CHCl_3$. | T.° during. | Resistance after $CHCl_3$. | T.° after. | Percentage of $CHCl_3$ vapour. | $R_3/R_1$. | Notes. |
|---|---|---|---|---|---|---|---|---|---|
| 201 A | 125,000 | 17·0° | 125,000 | 18·0° | 122,000 | 16·5° | — | 0·976 | Thread |
| B | 82,000 | | 88,000 | | 85,000 | | | 1·036 | Nerve |
| 202 A | 81,000 | 16·8 | 80,000 | 17·3 | 78,000 | 17·3 | 16·0 | 0·963 | Thread |
| B | 85,000 | | 90,000 | | 87,000 | | | 1·023 | Nerve |
| 203 A | 88,000 | 17·6 | 90,000 | 18·3 | 92,000 | 18·0 | 16·0 | 1·045 | Thread |
| B | 80,000 | | 82,000 | | 79,000 | | | 0·987 | Nerve |
| 204 A | 81,000 | 16·5 | 80,000 | 17·5 | 77,000 | 17·5 | 13·5 | 0·951 | Thread |
| B | 176,000 | | 176,000 | | 172,000 | | | 0·973 | Nerve |
| 205 A | 111,000 | 17·5 | 110,000 | 17·8 | 107,000 | 17·8 | 13·0 | 0·964 | Thread |
| B | 190,000 | | 199,000 | | 183,000 | | | 0·963 | Nerve |
| Mean of 5 thread Experiments (A) | 97,200 | 17·08 | 97,000 | 17·78 | 95,200 | 17·42 | — | 0·979 | |
| Mean of 5 nerve Experiments (B) | 122,600 | | 127,000 | | 121,200 | | | 0·989 | |

These figures show that—

(1) Nerves tested by Wheatstone's bridge show an apparent diminution of resistance after $CHCl_3$ of about 15 per cent.

(2) Nerves tested by the Kohlrausch method show no alteration of resistance.

The difference between the two methods is that any polarisation in the nerve appears by the first as an added resistance. The figures in the first column of A therefore represent resistance and polarisation, and the conclusions follow—

A. $CHCl_3$ diminishes the polarisation of nerve;

B. $CHCl_3$ does not alter the real resistance of nerve within the limit of error of the method.

If the diminution in the control experiments is taken as a correction to be applied to the nerves, the resistance of these after $CHCl_3$ is increased by 1 per cent., at present it is doubtful if this correction is to be applied.

[*Note added December* 12.—A fresh series of experiments (Nos. 211 to 214) gave the final ratio for nerves slightly *less* than the control threads. The probable limit of error of the resistance experiments in the text is about ±2 *per cent.*, and as this lies in the object examined rather than in the measurements, greater accuracy seems at present unattainable.]

### *Series VI.*

Similar experiments were made with ether, taking readings by both methods. The control threads now show a diminution of about 5 per cent. and an increase during etherisation. Except in this latter particular the nerves gave results almost identical with $CHCl_3$.

## Series VI.

| Experiment. | Resistance before ether, ohms = $R_1$. | T.° | Resistance during. | T.° | Resistance after, $R_3$. | T.° | $R_3/R_1$. | Percentage of ether. | Notes. |
|---|---|---|---|---|---|---|---|---|---|
| **A¹.—Control Threads. Ether. Wheatstone Bridge.** | | | | | | | | | |
| 162 | 71,700 | 18°·2 | 86,000 | ° — | 71,000 | 18°·0 | 0·990 | 40·0 | In this series the nerves and threads were measured successively, and the temperature was taken in the vapour of the ether, and not alongside the nerve as elsewhere |
| 165 | 122,400 | 18·8 | 143,000 | — | 110,400 | 18·4 | 0·893 | 50·0 | |
| 167 | 105,200 | 18·7 | 127,000 | — | 100,900 | 18·5 | 0·959 | 52·4 | |
| | 99,770 | — | 118,600 | — | 94,100 | — | 0·947 | | |
| **A².—Sciatic of Frog.** | | | | | | | | | |
| 161 | 156,600 | 17·9 | 169,300 | 1·0 | 129,300 | 17·9 | 0·826 | 55·0 | |
| 163 | 86,400 | 18·2 | 106,600 | — | 78,700 | 17·8 | 0·911 | 51·0 | |
| 164 | 165,800 | 19·0 | 190,000 | — | 141,300 | 18·8 | 0·852 | 47·2 | |
| 166 | 163,900 | 18·7 | 198,000 | — | 142,400 | 18·5 | 0·869 | 50·0 | |
| | 143,180 | | 165,980 | — | 122,910 | — | 0·865 | | |
| **B.—Frog's Sciatic and Thread. Kohlrausch. Ether at 50 per cent. approx.** | | | | | | | | | |
| 207 A<br>B | 74,000<br>68,000 | 18·4 | 98,000<br>109,000 | 18·8 | 71,000<br>73,000 | 18·6 | 0·960<br>1·073 | —<br>— | Thread<br>Nerve |
| 208 A<br>B | 90,000<br>85,000 | 18·5 | 118,000<br>129,000 | 18·6 | 83,000<br>85,000 | 18·5 | 0·921<br>1·000 | —<br>— | Thread<br>Nerve |
| 209 A<br>B | 66,000<br>72,000 | 17·8 | 83,000<br>103,000 | 18·2 | 63,000<br>69,000 | 18·2 | 0·955<br>0·958 | —<br>— | Thread<br>Nerve |
| 210 A<br>B | 103,000<br>77,000 | 18·2 | 132,000<br>116,000 | 18·5 | 99,000<br>73,000 | 18·3 | 0·961<br>0·959 | —<br>— | Thread<br>Nerve |
| Mean of 4 thread (A) | 83,300 | 18·2 | 107,800 | 18·5 | 79,000 | 18·4 | 0·949 | | |
| Mean of 4 nerves (B) | 75,500 | 18·2 | 114,300 | 18·5 | 75,000 | 18·4 | 0·994 | | |

These figures show—

(1) Ether diminishes the polarisation in nerve.

(2) Ether does not alter the real resistance of nerve (with the same reserve as to the control experiments).

Comparing the result of the two anæsthetics, and taking the mean figures:—

| | Mean resistance before. | Mean resistance after. | $R_3/R_1$. | |
|---|---|---|---|---|
| Nerve, $CHCl_3$ ............ | 121,400 | 103,500 | 0·854 | Wheatstone. |
| „ ether ............ | 143,180 | 122,910 | 0·865 | |
| Nerve, $CHCl_3$ ............ | 122,600 | 121,200 | 0·989 | Kohlrausch. |
| „ ether ............ | 75,500 | 75,000 | 0·994 | |

The final effects of $CHCl_3$ and ether are identical, within the limits of error of the experiments.

## *Observations.*

The full discussion of the problems raised by the facts here recorded must be deferred until the results are considered of experiments on other tissues and on the question as to the actual effect of the action of $CHCl_3$ and ether on proteid solutions.* Taken as they stand, the present experiments show:—

(1) That chloroform and ether (and probably alcohol) produce an electromotive effect when acting on a frog's nerve, which has a maximum value of about 0·030 volt, and the same sign as the current of injury.

(2) That $CHCl_3$ and ether produce no alteration of the resistance of the nerve (within the limits of error), but diminish the polarisation.

Two inferences present themselves—

A. That the electromotive effects are due to the same cause that produces the injury current.

B. That as the resistance is not diminished no additional ions are formed.

The discussion on the correctness or otherwise of these inferences is postponed for the reasons given.

I have again great pleasure in acknowledging the kindness of Dr. Waller, both for the permission to work in the laboratory of the University of London, and for advice in the conduct of the experiments. Also to express my appreciation of the assistance of Dr. B. J. Collingwood, in conjunction with whom many of the experiments were carried out. Mr. Shapiro has also given me much help in the later stages of the research.

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* See Moore and Roaf, 'Roy. Soc. Proc.,' and also Waller, *loc. cit.*

## APPENDIX. Protocols of Experiments in Series III.

The figures given are those of the potentiometer, balancing the current from the nerve at 30 sec. intervals.

| $CHCl_3$. | | | | | Ether. |
|---|---|---|---|---|---|
| Experiment 107. Electrode current $-3$. | Experiment 108. Electrode current $-6$. | Experiment 109. Electrode current $-8$. | Experiment 110. Electrode current $= +4$. | Experiment 111. Electrode current $-8$. | Experiment 117. Electrode current $-8$. |
| $-156$ | $-184$ | $-21$ $\frac{1}{1000} = 3{\cdot}3$ | $-176$ $\frac{1}{1000} = 3{\cdot}5$ | $-25$ | $-8$ $\frac{1}{1000} = 2{\cdot}0$ |
| $-154$ | $-182$ | $-21$ | $-175$ | $-26$ | $-9$ |
| 151 | 179 $\frac{1}{1000} = 2{\cdot}7$ | 21 | $-175$ | 26 | 10 |
| 149 | 177 | 22 no cut end | 175 | 27 | 11 |
| 148 | 175 | 23 | 175 | 27 no cut end | 11 no cut end |
| 146 T° = 17°·3 | 174 | 23 | 175 | 26 | 12 |
| 145 | 172 | 23 | 176 | 25 | 12 |
| 145 | 171 | 22 | 176 | 25 | 12 |
| 143 | 169 | $-22$ | 175 | 27 | $-12$ |
| $-141$ | 168 | $-22$ | $-175$ | $-25$ | |
| | $-167$ | | | | |
| $CHCl_3$ to long. surf. | $CHCl_3$ to long. surf. | $CHCl_3$ to prox. end. | $CHCl_3$ to cut end. | $CHCl_3$ distal end. | Ether to distal end. |
| $-120$ | $-144$ | $+20$ | $-190$ | $-90$ | $-120$ |
| $-80$ | $-114$ | $+50$ | $-218$ | $-120$ | $-200$ |
| $-45$ | $-84$ | $+78$ | 233 | 160 | 250 |
| $-20$ | $-50$ | 94 | 254 | 189 | 270 |
| $+8$ | $-20$ | 105 | 268 | 210 | 272 |
| $+25$ | $+7$ | 118 | 280 | 227 | 270 $\frac{1}{1000} = 1{\cdot}6$ |
| 35 | $+30$ | 130 | 290 | 238 | 269 |
| 44 | 46 | 132 | 296 | 243 | 267 |
| 52 | 60 | 142 | 302 | 247 | 267 |
| 58 | 67 $\frac{1}{1000} = 3$ div. | 146 | 304 | 249 | $-266$ |
| 63 | 75 | 150 | 306 | 250 | Ether off |
| 66 | 80 T° = 16°·8 | 148 | 307 | 252 | 5 mins. application only |
| 68 | 85 | 150 | 308 | 252 | |
| 70 | 88 | 153 | 308 | 257 | |
| 71 | 92 | 155 | 307 | 255 | |
| 72 | 94 | 158 | 306 | 255 | |
| 72 | 95 | 158 | 306 | 255 | |
| 73 | 96 | $+159$ | 305 | 255 | |
| 73 | 97 | $+160$ | 305 | 254 | |
| $+74$ T° = 17°·2 C. | $+99$ | | $-304$ | $-254$ | |
| $CHCl_3$ off | $CHCl_3$ off | $CHCl_3$ off | $CHCl_3$ off | $CHCl_3$ off | |

APPENDIX. Protocols of Experiments in Series III—*continued.*

| $CHCl_3$. | | | | | Ether. |
|---|---|---|---|---|---|
| Experiment 107. Electrode current −3. | Experiment 108. Electrode current −6. | Experiment 109. Electrode current −8. | Experiment 110. Electrode current = +4. | Experiment 111. Electrode current −8. | Experiment 117. Electrode current −8. |
| + 75 | + 98 T° = 16°·9 | +160 | −303 | −253 | −266 |
| 77 | 98 | 158 | 301 | 251 | 264 |
| 77 | 98 | 151 | 300 | 250 | 263 |
| 77 | 98 | — | 300 | 249 | 263 |
| 77 $\frac{1}{1000}$ = 3 div. | 98 | 157 | 300 | 248 | 263 |
| 78 | 98 | 157 | 299 | 248 | 262 |
| 79 | 98 | 157 | 299 | 247 | 262 |
| 80 | 98 | 158 | 299 | 246 | 261 |
| 82 | 98 | 158 | 298 | 246 | 261 |
| + 82 | + 98 | +158 | −298 | −246 | −260 |
| $CHCl_3$ to cut end. | $CHCl_3$ to cut end. | $CHCl_3$ to distal end. | $CHCl_3$ to long. surf. | $CHCl_3$ to prox. end. | Ether to prox. end. |
| + 40 | + 66 | — | −283 T° = 16·1 | −230 | −220 |
| + 20 | + 42 | + 58 | −255 | — | −150 |
| + 1 | + 20 | + 34 | −220 | −155 | 101 |
| − 10 | + 5 | + 10 | −180 | 130 | 60 |
| − 20 | − 5 | − 6 | −150 | 109 | 37 |
| − 22 | − 12 | − 17 | −123 | 87 | 23 |
| − 24 | − 16 | − 24 | −100 | 70 | 16 |
| 24 | − 18 | − 29 | − 83 | 57 | 11 |
| 23 $\frac{1}{1000}$ = 2·8 div. | − 18 | − 30 | − 73 | 46 | 8 |
| 23 | − 18 | 30 | − 62 | 40 | − 7 |
| 23 | − 17 | 29 | 54 | 34 | Ether off |
| 23 | 17 | 30 | 47 | 29 | 5 mins. application only |
| 23 | 17 | 29 | 44 | 25 | |
| 23 | 17 | 29 | 41 | 24 | |
| 23 T° = 17°·1 | 17 | 28 | 38 | 22 | |
| 23 | 17 | 28 | 36 | 20 | |
| 23 | 17 | 28 | 35 | 20 | |
| 23 | 17 | 28 | 35 | 19 $\frac{1}{1000}$ = 3·6 | |
| 24 | − 17 | 28 | 34 | 19 | |
| 24 | − 17 T° = 16°·8 | 27 | | − 19 | |
| $CHCl_3$ off | $CHCl_3$ off. | $CHCl_3$ off | $CHCl_3$ off | $CHCl_3$ off | |

| | | | | | |
|---|---|---|---|---|---|
| − 24<br>24<br>24<br>24<br>23<br>23<br>21 | − 17<br>17<br>17<br>17<br>17<br>17<br>− 17 | − 27<br>29<br>28<br>28<br>27<br>26<br>25<br>− 23<br>− 25 | − 32<br>32<br>31<br>31<br>30<br>30<br>− 26 | − 14<br>11<br>10<br>9<br>9<br>7<br>− 8 | − 6<br>− 6<br>6<br>8<br>9<br>9 |
| $CHCl_3$ to whole nerve.<br>− 20<br>22<br>22<br>Electrode current = −3 | $CHCl_3$ to whole nerve.<br>− 17<br>16<br>17<br>17<br>Electrode current = −4 | $CHCl_3$ to whole nerve.<br>− 33<br>− 33<br>33<br>33<br>Electrode current = −9, $\frac{1}{1000}$ = 2·8 | $CHCl_3$ to whole nerve.<br>− 28<br>26<br>25<br>24<br>Electrode current = −11 | $CHCl_3$ to whole nerve.<br>− 13<br>14<br>13<br>13<br>Electrode current −6 | Electrode current = −11 |

# *Further Work on the Development of the Hepatomonas of Kala-Azar and Cachexial Fever from Leishman-Donovan Bodies.*

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[Plate 7.]

In 1903 Lieutenant-Colonel Leishman, R.A.M.C., described certain bodies in the spleen of a fatal case of chronic fever in a soldier invalided home from near Calcutta, which he considered to be degenerate trypanosomes, on account of their resemblance to the breaking-up dead trypanosomes found in the spleens of rats 48 hours after death, and he therefore suggested that trypanosomes might be present during life in this class of fever. Major Donovan, I.M.S., (2) of Madras, however, very shortly after showed that Leishman's surmise was not correct, as he found similar bodies to those described by Leishman in fresh blood obtained by puncturing the spleen during life, but no trace of trypanosomes; and after examining Donovan's specimens, M. Laveran (3) pronounced the parasite to be a Piroplasma, and suggested the name *Piroplasma Donovani* for them.

Donovan (4) also claims to have found the parasites in the red corpuscles of the peripheral blood, but his coloured illustrations of them very closely resemble ring-parasites of malaria, and have only one chromatine body, and his statements in this respect have not been confirmed by any other observer. Ross (5) suggested that the parasite probably belonged to a new genus, and proposed to call it *Leishmania Donovani*, and Manson and Nuttall also favoured the view that it is a distinct genus. In the following year, 1904, on my return to India from leave, I commenced an investigation of the subject, with a view to finding further stages of the life history of the parasite which might throw light on its true nature and classification. In the meantime Lieutenant Christophers, I.M.S., had been placed on special duty by the Government of India to investigate the subject, and after making a careful study of the parasite in different tissues of the body, he suggested that they might be spores of a microspordium. (6)

My first endeavour was to find some method of keeping the parasite alive outside the human body, and after a number of trials success was obtained by preserving spleen blood containing the parasites under sterile conditions in a cold incubator, preferably at about 22° C. For this purpose the fresh blood obtained by spleen puncture during life was placed in small sterile test-tubes containing a few drops of 2 to 5 per cent. citrate of soda in normal salt solution, in order to prevent clotting. Under these conditions not only did the parasites remain alive for many days, but they also multiplied very rapidly, became much enlarged, and after about three days some of them developed into elongated flagellated bodies, which I took to be a stage in the development of a trypanosome, although no undulating membrane was yet present. This discovery was announced in the 'Lancet' of July 23rd, 1904, a few uncoloured illustrations were published in September, (7) and a fuller paper tracing the stages of the development day by day, with a coloured plate, in November (8) of the same year. Confirmation of my discovery was first furnished by my assistant, Dr. G. C. Chatterjee, (9) working in my own laboratory, and next independently by Lieutenant Christophers, (10) working in Madras. Thirdly, Captain Statham (11) and Lieutenant-Colonel Leishman also obtained the development, and published their account of it, March, 1905.

During the past year I have made a large number of experiments on the conditions affecting the development of the parasites outside the human body, with a view to obtaining a clue to the natural mode of infection, and early in the present year I published a summary of the results obtained. The most important conclusions arrived at were, that sterility is essential to the continued development, and that flagellation takes place much more uniformly and regularly if the citrated spleen blood is faintly acidified with citric acid: facts which strongly point to the stomach of some blood sucking insect as the natural place of development of the parasite outside the body, and I gave some clinical reasons for considering the common bed bug to be the most likely conveyer of the disease. So much more abundant development of flagellated stages has recently been obtained by the use of acidified blood medium, that I have been able to make a more satisfactory study of the exact mode of development, and to come to a definite conclusion regarding the ultimate stage it reaches, and therefore propose to describe and illustrate these later stages more fully than in my previous papers, and to briefly discuss the bearing of the conditions affecting the development of the flagellated stage of the parasite on the probable mode of infection of the disease.

### *Stages of Development of the Parasites observed in Acidified Citrated Blood.*

In the first place, the development in acidified blood is much more uniform than that obtained by the previous method, so that instead of finding all stages present after three or four days, with a great preponderance of the smaller oval forms, and but few flagellated ones as in alkaline blood, in the acid medium the great majority of the parasites will be found in nearly the same stage on any given day, and nearly all become flagellated after a few days. The sequence of events during the first two days is the same as I have already described, (8) and they are well shown in the first two lines of the accompanying plate—all the figures in which have been drawn to the uniform scale of 1500 diameters magnification, with the aid of a camera lucida. Line I shows the parasites seen in a film of spleen blood made at the time it was obtained, and consequently before any development had taken place. After incubation for two days at 22° C. the forms shown in Line II were present, figs. 1 and 2 showing considerable enlargement, especially of the macronucleus and protoplasm of the body. Figs. 3 and 4 also show the earliest appearance of the eosin-staining body, which is represented as a clear space in the drawings, but is of a rosy-pink colour with Romanosky's stain, and quite distinct from the vacuoles, the latter being indicated by the more lightly shaded portions of the protoplasm. It will also be noted that from the first the micronucleus, or blepharoplast, is closely attached to the eosin body (called by Leishman "flagellar body").

Further, on the second day in this acid culture a few of the early flagellated forms shown in figs. 7 and 8 of Line II were also seen, although they do not usually appear in alkaline cultures until at least the third day, while just antecedent to this stage are the forms shown in figs. 5 and 6 of the same line, illustrating commencing elongation and division by fission, and it will be observed that in these the eosin body is passing up to the anterior end of the organism from which the flagellum arises, and is carrying the micronucleus with it. In my earlier description I suggested that the double elongated forms shown in fig. 6 of this line might possibly represent a form of conjugation preparatory to the development of the flagellated stage of the organism, but further study of a much larger amount of material has convinced me that they are only fission forms, as I have been unable to make out any reduction in the number of chromosomes in the macronucleus during the process.

### *The Mode of Division of the Flagellated Forms and the Formation of Rosettes.*

In Line III of the plate are represented the different stages of division of newly formed flagellated bodies. Figs. 1 and 2 show that the micronucleus and flagellum first divide, just as in trypanosomes, and next the macronucleus divides in turn, and a clear line appears in the length of the organism, indicating commencing division of the protoplasm of the body, as shown in figs. 3 to 5 of the same line; while in fig. 6 the division of the body has just been completed, and in fig. 7 the micronuclei and flagella of a still adherent pair are dividing over again, thus showing how rapidly the multiplication was taking place in this culture, for such forms were not uncommonly seen in it.

In my earlier cultures the flagellated pairs were nearly always found in pairs only, although rarely three or four might be seen side by side. In the much more abundant development of flagellates in the acid culture medium, however, considerably larger masses, forming beautiful rosettes, with the flagella crossing each other in the centre, were seen in large numbers, and it is easy to understand how they may be formed by the rapid multiplication just described. Thus fig. 6 of Line IV shows a small group of flagellates which is remarkable for including nearly all the stages of division in a single clump, while fig. 9 shows the commencement of the formation of a rosette by the rapidly dividing flagellates pushing each other round to form a semicircular mass, and in fig. 12 is shown a small, but complete, rosette, several of the forms in which are undergoing further subdivision. In this stage the contents of the eosin bodies frequently becomes protruded, as I have previously noted, and it accumulates round the flagella, helping to bind the forms together into the rosette shape. Next, the individual organisms elongate, and at the same time become narrower, and the rosette then commences to break up, in consequence of the increasing motility of the flagella, and some now separate from the mass in pairs or single forms as indicated in fig. 11, and in this manner the free swimming forms shown in Lines IV and V of the plate are produced. In fresh specimens these are very active, the single ones in particular threading their way rapidly among the red corpuscles, and on reaching an open space, dart about in such a manner as to leave no doubt in the mind of the observer that the object of this remarkable development and extraordinary increase in size is to endow the motionless human stage of the organism with the power of locomotion required in some period of its extra-corporeal existence.

### *The Nature of the Fully Developed Flagellate Form of the Organism.*

When I first obtained the development of the flagellated stage, I thought them to be young trypanosomes which had not yet formed an undulating membrane. In support of this possibility, the recent observations of Novy and MacNeil (12) on the culture of trypanosomes of birds on blood agar are of great interest, for they obtained forms, separate and in rosettes, most closely resembling those shown in the plate accompanying the present paper, both in the absence of all trace of undulating membrane and in the position of the micronucleus or blepharoplast at the anterior flagellated end of the organism, although in addition they obtained forms showing the development of the membrane by the passage of the blepharoplast back towards, and then past, the macronucleus, until it arrived near the posterior end of the organism, and a typical trypanosome resulted. When further experience of my culture failed to reveal any forms with a complete or even partial undulating membrane, the question arose whether it was not an organism distinct from the trypanosomes, although closely related to it, such as a hepatomonas in which no undulating membrane is present. In my last paper I left this an open question, while stating that nothing had yet been found which might not be an incompletely developed trypanosome; a view which has also been adopted by both Christophers (10) and Leishman (11). The more abundant and uniform development of flagellates in the acidified medium have enabled me to study closely innumerable apparently completely developed long free forms, in a stage in which they show extremely active movement in fresh specimens; but still no trace of an undulating membrane, or even a tendency for the micronucleus to pass away from the anterior end of the organism towards the macronucleus has ever been observed, although seen by Novy and MacNeil in their cultures of bird trypanosomes. I therefore conclude that the organism I have been able to develop belongs to the order Hepatomonas and not to the trypanosomes, and I propose to name it the *Hepatomonas of Kala-azar*. At the same time I prefer to limit the term kala-azar to the epidemic-spreading form of the disease as seen in Assam, and to retain the term "cachexial fever" for the less fatal sporadic affection, if only for the sake of avoiding the unnecessary cruelty of having to tell sufferers from the milder disease that they are suffering from the greatly dreaded kala-azar.

### *Degenerate Forms.*

I have already pointed out that the absence of bacteria is necessary for the continued development of the flagellated stage of the organism, and that cocci especially are inimical to its growth. In one of my most active recent cultures staphylococci gained access to the tube on the seventh day of the culture during its repeated examination, and the degenerating changes resulting were readily followed. On the following day fresh specimens showed that all motion of the flagella had ceased, although on staining many of the organisms showed little or no change. Others, however, were granular and stained more lightly, while some were becoming shorter and more oval or pear-shaped, and their flagella shorter, as in figs. 2 to 6 of Line VI, thus showing a tendency to reversion towards the undeveloped spleen stage of the parasite, only all stages of the degenerative process were present at the same time, and many of the shrunken badly staining forms were disintegrating. During this process the flagella were often shed, and with it the micronucleus came away, although a narrow non-staining space was still visible between the two, as shown in fig. 10, clearly proving an organic connection between the flagellum and the micronucleus or blepharoplast. Within three days all the rosettes of flagellates had broken up into granular masses and their identity completely lost. The degenerative changes in this hepatomonas are therefore very similar to those which have been described in the case of trypanosomes.

### *The Relationship of Leucocytes to the Parasites in Cultures.*

Although it is doubtful as yet whether the Leishman-Donovan bodies can be found in the peripheral blood either free or in the red corpuscles, yet both Donovan (4) and Christophers (13) have found this stage of the parasite within leucocytes in the circulating blood during high fever, the latter having twice found a number of them, nearly all within polymorphonuclears, during a differential count of 500 leucocytes, which would mean an enormous number within the peripheral circulation at one time, and amply sufficient to infect a blood-sucking insect if such proved a suitable host. It is therefore of interest to determine if the parasites can develop in acid cultures within leucocytes. Figs. 7 and 8 illustrate conditions bearing on this point, the former representing a polymorphonuclear on the second day of the culture, which contains typical parasites, although they are somewhat less developed than those shown beneath it from the same slide; the latter shows another degenerating leucocyte from the same culture on the following day, in which some of the parasites are clearly much enlarged and developing typically, if somewhat more slowly, than those show outside the corpuscle, while others are degenerating and staining feebly. It appears then that development may proceed within leucocytes, while Christophers is also of opinion that it occurs within macrophages in cultures, so biting insects might be infected by the leucocytes containing the undeveloped parasites which have been found in the peripheral blood.

The way in which the polymorphonuclears especially take up the parasites in the peripheral blood is also of great interest in connection with the extreme decrease in these corpuscles, for I have shown that they are commonly decreased a tenth of the normal number, while in the latter stages of the disease, in children especially, they may fall to only from one-twentieth to one-sixtieth, thus readily accounting for the frequency of terminal infection by such diseases as dysentery, cancrum oris, pneumonia and phthisis, owing to loss of phagocytic power, while I have also found the opsonic index reduced against the staphylococcus *pyogenes aureus*, which is frequently present in the spleen in cases of cancrum oris.

### *The Bearing of the Flagellation of the Parasite in Sterile Acid Medium on the Probable Mode of Infection.*

The two factors which I have found most essential to a uniform development and very rapid multiplication of the flagellated forms are sterility and a slightly acid, or, at least, a neutral medium. I have also tried blood agar after Novy's method, only using human blood in its preparation, but failed to obtain either sub-cultures of already developed flagellates or of the spleen parasites, while only very scanty development was obtained when several drops of spleen blood, with very numerous parasites, were added to a previously acidified blood agar tube, and then only in the added blood as by the ordinary method. Now the only condition under which the Leishman-Donovan bodies would be likely to meet with a sterile acid medium on their escape from the human body would be in the stomach of some blood-sucking insect, of which the common bed bug, or possibly mosquitos, are the most likely hosts, for clinical reasons I have elsewhere pointed out, while I have found that after sucking blood it becomes acidified in gastro-ntestinal tract of bugs, and is also frequently sterile. I have not yet succeeded in inducing these insects to suck infected spleen blood placed in capsules of various kinds, but, on the other hand, I have mixed the contents of their stomachs after feeding on human blood (which was proved to be free from anything resembling any stage of the Hepatomonas of kala-azar) with about an equal quantity of spleen blood containing the parasites, and, after incubating in capillary tubes at 22° C., have been able to watch the development of the parasites day by day up to the flagellated stage under these conditions in those which remained sterile, but not when any bacteria were present. It is therefore clear that the conditions met with in the stomachs of bugs—and possibly also of mosquitos—are not inimical to the development of the parasite into the flagellated stage, provided the temperature conditions are suitable.

The more difficult question whether opportunities for infection of such insects occur sufficiently frequently to account for the incidence of the disease remains to be considered. In the first place, it is conceivable that bugs especially might become infected from skin lesions containing the parasites, for these may occur on parts of the body little exposed to the bites of mosquitos, but, in my experience such skin affections are too rare to alone account for the frequency of infection. Further consideration will, I think, show that the difficulty in finding these minute parasites in the peripheral blood does not necessarily exclude the possibility of their occurring there in sufficient numbers to infect insects, especially during high fever, when they have been found in circulating leucocytes. In the first place, it has been shown, by Christophers especially, that the organisms multiply in the endothelial cells lining the blood-vessels of the internal organs, such as the spleen, liver, and bone-marrow, and when numerous, in films obtained by spleen puncture, they are frequently seen in groups in fragments of these cells, which during life must frequently rupture and set them free in the circulation, as is also proved by the same observer having found them in the blood of some of the large veins. It is further of interest to note that the endothelial cells of these very same organs are the principal sites of the deposits of malarial parasites in the internal organs, while I have also several times found Leishman bodies in the brain (where malarial parasites also occur), so that it is clear that they must frequently enter the circulating blood in considerable numbers. Secondly, the human stage of the parasite is so small that it would be scarcely easier to find in the blood by microscopical examination alone that typhoid bacilli in that disease, although the latter can be readily obtained by cultural methods.

The great difficulty of finding the human stage of the Hepatomonas of kala-azar in the blood, even if present in sufficient numbers to infect suitable insects, is well shown by Novy and MacNeil's (12) experience of searching for trypanosomes in birds; for while they only succeeded in detecting this large actively moving parasite by microscopical examination of thick blood films in 8 per cent., nevertheless they cultivated the parasite on their blood-agar medium in 50 per cent. of the same series. Moreover, even when they found them by their movement in thick fresh films, yet in the same birds they frequently failed to detect them in stained specimens. How much more difficult would it be to demonstrate the minute motionless Leishman bodies, which can be only seen in thin stained films, even if they were present in relatively large numbers in the peripheral blood?

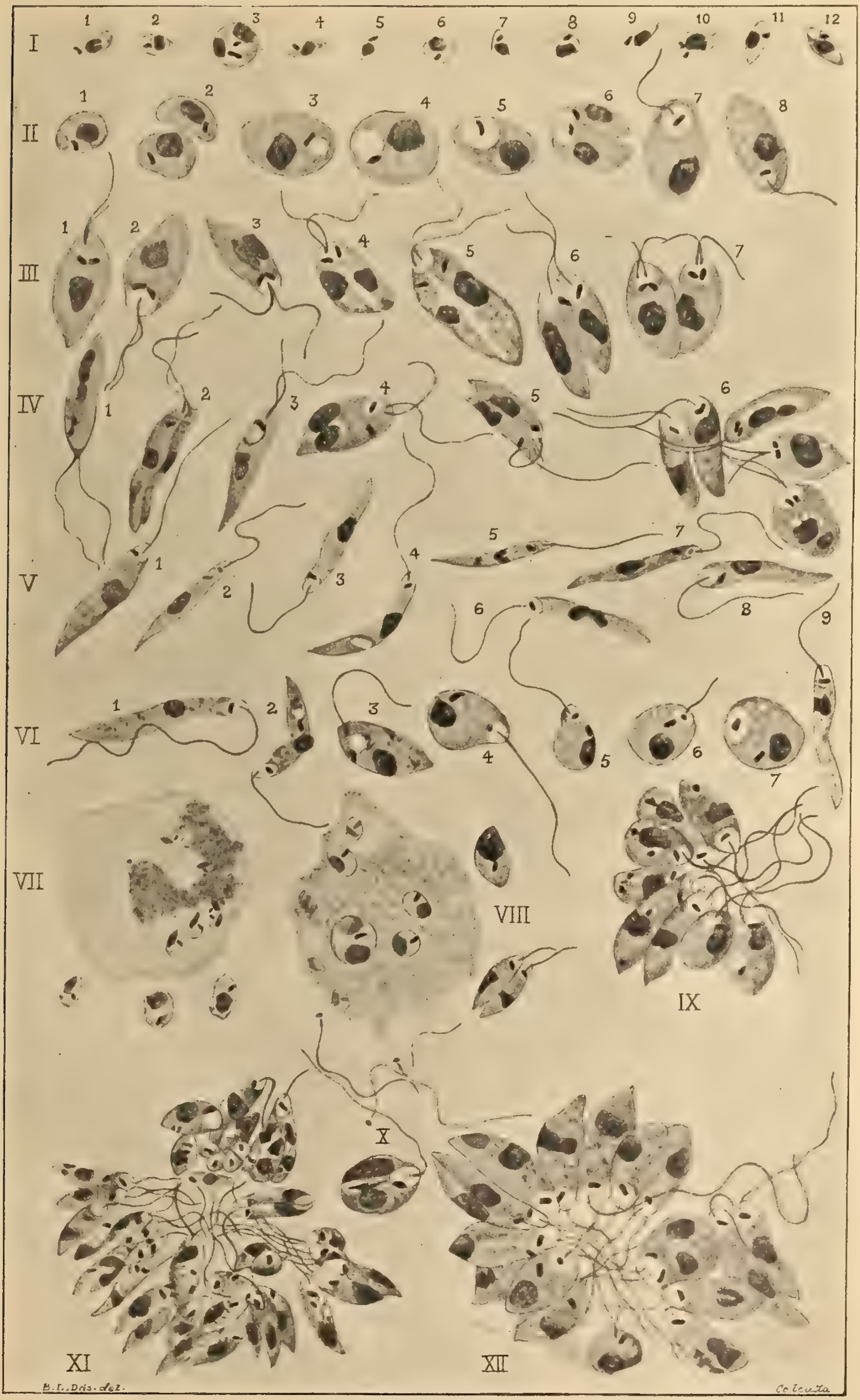
Thirdly, the extremely rapid multiplication of the flagellated forms in some of my recent cultures would appear to indicate that, in the presumably still more favourable natural conditions of the extra-corporeal stage of the parasite, a very small number of the human organisms would multiply to such an extent as to constitute a powerful infective agency.

The only reasonable alternative to the hypothesis just set forth is the suggestion of Manson, Christophers, and others, that the organism may escape from the body by means of ulcers sometimes found in the intestines, the granulation tissue of which contains the parasites, and they may thus reach water. Apart from the great rarity of such infected intestinal lesions in my very extensive *post-mortem* experience of this disease in Assam and Calcutta, the fact that sterility is essential for the continued development of the flagellated stage of the organism appears to me to make this mode of infection an exceedingly improbable one. Moreover, I have been unable to obtain any development of the organism in even sterile water kept at the most favourable temperature, while even in sterile acidified water similar negative results have recently been obtained.

### *Relationship of the Optimum Temperature for the Development of the Flagellates to the Seasonal Incidence of Kala-Azar and Cachexial Fever.*

If the conditions I have found necessary for the development of the flagellate stage of the Hepatomonas of kala-azar afford any indication of the natural conditions under which it occurs, then the striking fact that the relatively low temperature of about 22° C., or 72° F., is essential to the process, would indicate that infection is only likely to take place in India during the colder part of the year. Owing to the fever in this disease lasting for many months, or even several years, with long intervals of little or no rise of temperature, while cases not infrequently begin very insidiously, patients presenting themselves with marked, but often unsuspected, enlargement of the spleen, and a history of only a few days' fever; it appears probable that the incubation period may be a long one, and the onset very insidious and indefinite. Nevertheless a clear history can often be obtained, and an analysis of the notes of a number of cases showed five times as many in which the symptoms first commenced in the six months from November to April as in the remaining six hot months of the year, so that the cold weather months, together with the very commencement of the hot weather, to allow for the probable incubation period, show a very marked preponderance of the infection. Moreover, Dr. Dodds Price, of Assam, informs me, as a result of his unique experience of kala-azar, extending over 15 years, that every case he has seen in Europeans began in the cold season, and that among his hundreds of native cases he has noticed the same marked tendency for definite symptoms of the disease to first show themselves at that time of the year. The practical importance of this point in relation to the prevention of the disease is evident, while the close agreement of its seasonal incidence with the deductions from my experimental data is of considerable interest. It is also worthy of note that this disease is most prevalent in just those parts of India where the temperature conditions for

*Rogers.* *Roy. Soc. Proc., B. vol. 77, Plate 7.*


I
1 2 3 4 5 6 7 8 9 10 11 12
II
1 2 3 4 5 6 7 8
III
1 2 3 4 5 6 7
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X
XI
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Calcutta

several months of the cold season most closely correspond with that which I have found to be most favourable to the development of the flagellated stage of the Hepatomonas of kala-azar, namely Assam, Bengal, and Madras. On the other hand, the disease is much rarer, or has not yet been proved to originate, in those parts of India where the winter season presents a greater degree of cold, and the more favourable spring and autumn are very short.

Much work will be necessary to test the truth or otherwise of the above hypothesis, but knowledge should mean power to prevent the most terrible of all tropical diseases in its combined very high mortality and slow death by inches, and as the most favourable cold weather working season is approaching, it appears to be advisable to put these observations on record for the benefit of other workers in this very important field of tropical medicine.

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## DESCRIPTION OF PLATE.

Magnification of all the figures 1500 diameters.

FIG. I.—Undeveloped Leishman-Donovan bodies from spleen puncture film.
,, II.—Early stages of development, from two days' culture in acidified citrated blood; 1 and 2, body and macronucleus enlarged; 3 and 4, first appearance of eosin body; 5 and 6, elongation and subdivision; 7 and 8, first appearance of flagellum.
,, III.—Stages of division of the early flagellated forms.
,, IV.—Double long swimming forms.
,, V.—Fully developed long, free, active single cells.
,, VI.—Degenerate forms.
,, VII.—Undeveloped forms in a white corpuscle.
,, VIII.—Early stages of development in a degenerating white corpuscle.
,, IX.—Stage in the formation of rosette.
,, X.—Separated flagella with micronuclei attached.
,, XI.—Rosette breaking up into free forms.
,, XII.—Small complete rosette.

# *The Factors which Determine the Production of Intraocular Fluid.*

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In spite of the very numerous researches which have been made during the last half century on the seat and mechanism of production of intraocular fluid, ophthalmologists and physiologists are still far from an agreement on the subject, and a review of the literature reveals many discrepancies in the experimental evidence which it is impossible to clear away without a re-examination of the whole subject. The following paper contains the results of experiments made with the view of determining the weight to be ascribed to different experimental investigations.

As to the seat of production of the intraocular fluid, nearly all authorities are agreed that it is produced by the ciliary processes. From these processes a minute proportion travels backwards into the vitreous cavity, to be absorbed by the lymphatics of the optic disc, while by far the greater part makes its way between the lens and the ciliary processes, through the fibres of the suspensory ligament, into the posterior chamber, whence it passes round the margin of the iris into the anterior chamber. In addition to this mode of production, it has been suggested by Ehrlich that an appreciable amount of intraocular fluid may be secreted directly into the anterior chamber by the anterior surface of the iris. The experiments of Ehrlich (1) were made by the injection of a diffusible substance, fluorescine, and we agree with Leber (2) in regarding them as proving the possibility of diffusion between the vessels in the iris and the anterior chamber, but not the secretion of a normal intraocular fluid by this channel. At any rate, any fluid formed in this way is negligible when compared with that which is produced in the neighbourhood of the ciliary processes.

On the other hand, the place of absorption of the intraocular fluid is universally agreed to be the angle of the anterior chamber. Here the fluid is passed under pressure into the spaces of Fontana, whence it makes its way into the canal of Schlemm, between the endothelial cells lining this canal, and so is carried away into the venous system. This absorption is continuous, and its rapidity is largely determined by the height of the intraocular pressure. Since we have a constant absorption and a constant pouring out of fluid into the eyeball, it is evident that the intraocular pressure must be a product of the two factors, formation and absorption, and that the maintenance of the pressure at a constant height must be determined by an accurate balance between these two processes. The problem which lies before us is to determine the mechanism of formation of this fluid.

The intraocular fluid is a clear, colourless solution containing a proportion of salts similar to that of the blood plasma, but having an osmotic pressure which is somewhat higher than the blood plasma, and containing the merest trace of proteids.*

## I. *Methods of Research.*

The animals used were mostly cats. In a few cases dogs were employed, and in one experiment a rabbit. In the case of the cats the anæsthetic used was always ether, with the addition in some cases of a small dose of morphia. In a few experiments, after the induction of full anæsthesia, a small dose of curare was given. The administration of the anæsthetic was continued during the experiment by an air-pump connected with a cannula in the trachea. For the dogs the A.C.E. mixture was employed.

A record of the blood pressure was kept in all experiments. In some it was taken continuously, but in the greater number of experiments a short record was taken every few minutes in order to avoid trouble with clotting in the cannula. In the cats the blood pressure was taken in the lower part of the abdominal aorta, in the dog in the femoral artery.

The apparatus we employed for measuring the intraocular pressure was very similar to that described in a former paper (3). A graduated tube with internal bore of about 0·5 mm., and about 50 cm. long, is provided with a lateral tube near each end. One end of the tube is connected by india-rubber tubing, by means of a T-piece, with a reservoir containing Ringer's solution (or any other fluid the absorption of which is to be determined), and also with a manometer. The other end is connected by a second (glass) tube with a gilt steel hollow needle, which is introduced into the anterior chamber of the eye. The needle may be open at the end, or be closed at the end and provided with a lateral opening. To each of the side tubes a rubber capsule is attached. The capsule nearest the reservoir contains air, while that towards the eye is filled with fluid. By means of screw-clamps, fluid or air may be driven from either of the two capsules into the graduated tube. Before introducing the needle into the anterior chamber, the pressure in the apparatus is adjusted by raising the reservoir to about 25 cm. $H_2O$, which represents the average intraocular pressure. While the fluid is dropping from the end of the needle, this latter is thrust through the lateral part of the cornea, so as to lie in the middle of the anterior chamber. A bubble of air is introduced into the graduated tube by compression of one capsule, and brought to the middle of the tube by relaxing the clamp on the capsule at the end towards the eye. The reservoir is then rapidly adjusted to such a height that the bubble remains stationary.

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* Full details of various analyses of intraocular fluid are given by Leber (2), p. 207, *et seq.*

In some of the later experiments a platino-iridium cannula, with a solid steel point made slightly conical, was found to be an improvement, as, in the event of any leaking occurring, it could be pushed in further.

In introducing the cannula great care must be used, as, should the needle catch in or tear the iris, or wound the lens, the eye would be rendered useless for the purposes of the experiment. The needle, being comparatively large and blunt, requires considerable force for its introduction. We have found it safer to make a small perforation with the point of a cataract knife and, without letting the aqueous humour escape, to introduce the cannula in the hole thus made. Should the exact spot be lost sight of, a little fluorescine will stain it. A fine silk thread passed through the episcleral tissue, as in the operation for advancement of a rectus tendon, gives a better hold than fixation forceps, and is somewhat less in the way.

The fluid employed in the apparatus was usually Ringer's solution, in some cases normal saline. Whichever fluid was employed, it was filtered through a Berkefeld candle before the experiment, in order that no foreign body might be present which could lodge in and block the filtration channels.

The intraocular fluid must play a twofold function in the eye. In the first place, by keeping up the intraocular pressure, it lends rigidity to the supporting structures of the eyeball, and furnishes therefore a fixed point for the intraocular muscles to contract against, besides maintaining the proper distances between the various refractive media. In the second place, it is the only source of nourishment to certain of the structures of the eye, namely, the middle and back part of the cornea, the lens and suspensory ligament, and the vitreous humour. The question that we have to decide is whether this fluid is formed by a process of secretion by the cells covering the ciliary processes, or whether it is a transudation similar to lymph. The question presents many analogies to that with regard to the secretion of urine. In each case we have a possible source of transudation in the capillary blood-vessel network and also an absorbing mechanism. We can only arrive at a conclusion by determining the physiological conditions under which we may alter either the production or the absorption of the intraocular fluid.

## II. *The Effect of Changes in the Circulation on the Formation of Intraocular Fluid.*

If the production of intraocular fluid is dependent on a process of filtration through the blood vessels and the epithelium covering the ciliary processes, its rate must vary directly with the difference of pressure on the two sides of the filtering membrane. It must vary, therefore, directly with changes in the capillary blood pressure, and inversely with the changes in the intraocular pressure. In our first series of experiments we sought to eliminate the second factor, namely, that of absorption, by opening the anterior chamber, so that the intraocular pressure could be regarded as zero. A cannula was introduced into the anterior chamber and the fluid allowed to flow off into weighed porcelain capsules. These were changed every 10 or 20 minutes, and the amount of fluid secreted in the time determined by weighing. The fluid drained off during the first minute after insertion of the cannula was regarded as normal intraocular fluid, but the gradual emptying of the eye-ball continues during the first five minutes, so that the figures obtained during this time cannot be regarded as expressing the rate of secretion. In every case the total solids of the intraocular fluid were also determined.

The following experiment, p. 298, shows the results obtained while the blood pressure was approximately constant. It will be seen that there is a constant diminution in the amount of fluid obtained. In these experiments we were at first troubled by the formation in the anterior chamber of clots, which tended to plug the cannula. We found that this difficulty could be obviated by the injection of a dose of leech extract, not large enough to cause a permanent diminution of the blood pressure.*

The next question to determine was whether it was possible to alter the rate of production or the composition of the intraocular fluid by altering the blood pressure in the vessels of the eye-ball. The experiments on this point were all carried out on dogs. A diminution of the intraocular blood pressure was easily effected by ligature or obstruction of the carotid artery on the same side. In order to produce a maximal rise of pressure in the blood-vessels of one eye, the vertebral and subclavian arteries on both sides were tied. A loose ligature was placed round the thoracic aorta, so as to permit of its being obstructed at any given time. A cannula connected to the mercurial manometer was placed in the carotid artery on the right side. The production of intraocular fluid was determined in the left eye. By obstruction of the aorta a large rise of blood pressure was produced in this eye, since all the blood had to pass through the one carotid artery in order to get back to the heart. On the other hand, an almost complete anæmia could be produced in the eye by obstruction of the one remaining carotid. We give below the results of one such experiment.

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* This procedure had been previously employed by Mr. E. Pflüger in some experiments carried out in this laboratory in 1902. An account of these experiments will shortly appear as a dissertation in the University of Bern.

Cat, anæsthetised with Ether and the A.C.E. Mixture. A small dose of Curare was injected after anæsthesia was complete. The extract of 2 grammes of dried leech heads was injected.

| Time. | B.P. in mm. Hg. | Weight of secretion. | Weight of solids after drying to a constant weight. | Percentage of solids. | Rate of flow per minute. |
|---|---|---|---|---|---|
| | | grammes. | grammes. | | grammes. |
| 3.50 cannula inserted. | | | | | |
| 3.51 ........................ | 130 | 0·689 | 0·009 | 1·3 | |
| 3.56 ........................ | 145 | 0·252 | 0·007 | 2·7 | 0·05 |
| 4.16 ........................ | 120 | 0·756 | 0·032 | 4·2 | 0·037 |
| 4.36 ........................ | 100 | 0·475 | 0·021 | 4·4 | 0·023 |
| 4.56 ........................ | 96 | 0·482 | 0·024 | 4·9 | 0·024 |

Dog. Weight, 7½ kilos. Anæsthetised with the A.C.E. mixture and morphia. The extract of 2 grammes of dried leech heads was injected. Both subclavians and vertebrals were tied. Temporary ligature round aorta. Cannula in left eye. B.P. observed in right carotid.

| Time. | B.P. in mm. Hg. | Amount of secretion in grammes. | Total solids in grammes. | Percentage of solids. | Rate of flow. | Remarks. |
|---|---|---|---|---|---|---|
| 3·29 | — | — | — | — | — | Cannula inserted. Aorta unobstructed |
| 3·30 | 110 | 0·811 | 0·013 | 1·5 | — | |
| 3·35 | 110 | 0·432 | 0·014 | 3·2 | 0·086 | |
| 3·45 | 100 | 0·550 | 0·027 | 4·9 | 0·055 | |
| 3·55 | 205 | 1·153 | 0·068 | 5·9 | 0·115 | Aorta obstructed. Fluid tinged red. |
| 4·5 | 100 | 0·627 | 0·039 | 6·2 | 0·062 | Aorta unobstructed. |
| 4·15 | 198 | 0·816 | 0·053 | 6·6 | 0·081 | Aorta obstructed. |

It will be seen that in every case a rise of intraocular pressure caused an increase in the amount of fluid secreted. It is impossible, however, to deduce directly from these experiments that the intraocular fluid is a transudation. The opening of the eye-ball and the consequent diminution of the intraocular pressure to nothing have a serious effect on all the intraocular structures.

Great dilatation of the vessels of the ciliary processes and iris is produced. The fluid, which, in the normal eye, is free from fibrinogen and contains the merest trace of proteid, rapidly acquires the power of coagulation, and its proteid content rises to 3, 4, or 5 per cent. The serious alteration of the vascular structures is shown in many cases by the appearance of red blood corpuscles in the fluid dropping from the cannula, and Greeff has shown that if the lowered pressure be brought about suddenly and maintained for some time, the epithelium covering the ciliary processes may be raised from the surrounding tissue so as to form small blisters, which are filled with coagulable lymph. It has been suggested by Greeff (4) that the change in composition of the intraocular fluid ensuing on opening the eye-ball is determined by the separation of the epithelium, but Bauer (5) has shown that the proteid contents may be raised in the absence of these epithelial changes, and that, on the other hand, the epithelial changes may be well marked on the subsequent day, when the wound in the cornea has closed, and the intraocular fluid has regained its normal composition. He also points out that the amount of change produced depends entirely on the rapidity with which the intraocular pressure is lowered. The change in composition is probably due, as Leber suggests, to the great distension of the capillaries and the consequent separation of their endothelial cells. It represents in fact an alteration in permeability of the filtering membrane.

### III. *Amount of Intraocular Fluid Produced under Normal Circumstances.*

In any investigation of the factors determining the production and absorption of intraocular fluid, it is important to get some idea of the amount of this fluid secreted under normal circumstances, that is at normal intraocular pressure. Since the intraocular pressure is maintained constant so long as the blood pressure is steady, the amount of fluid produced at a given intraocular pressure must be equal to the amount of fluid absorbed at the same pressure. It is therefore a matter of indifference whether we measure the amount formed or the amount absorbed at any given pressure. Le Plat (6) sought to abolish the absorption of the intraocular fluid by filling the anterior chamber with oil or vaseline. A cannula was placed in the vitreous cavity, and the pressure in the cannula maintained at the normal intraocular pressure. It was found that the obstruction of the absorbing angle of the eye-ball carried out in this way caused a rise of intraocular pressure if the eye-ball were closed, or a flow outwards of intraocular fluid by the cannula if the pressure in this was maintained at the normal intraocular pressure. The amount of this outflow was measured, and was regarded by Le Plat as representing the normal rate of formation of intraocular fluid. He arrived at the conclusion that the amount of fluid normally secreted by the ciliary processes is in the rabbit about 4 c.mm. per minute. We found considerable difficulties in applying this method, chiefly determined by the tendency of the cannula in the vitreous to become blocked. We therefore adopted a method similar to that already employed by Niesnamoff, (7) under Leber's direction. The arrangement of the experiment was as follows:—

The hollow needle, connected by the capillary tube (containing an air bubble as index) to the reservoir and manometer, was introduced into the anterior chamber. The height of the reservoir was then adjusted until the bubble was stationary, showing that the intraocular pressure was exactly balanced by the pressure of the fluid in the tube leading to the reservoir. This intraocular pressure was of course maintained by a constant secretion of intraocular fluid, exactly equal to the amount escaping by filtration through the anterior angle of the eye. The animal was then killed by dividing the heart. This procedure at once stopped the production of intraocular fluid. The intraocular pressure, however, was maintained at its previous height by the connection of the eye with the reservoir of Ringer's fluid: the escape fluid by the anterior angle was therefore the same as before. The rate of this escape could be determined by noting the rapidity with which the air bubble moved along the capillary tube towards the eye, and this rate must be equal to the rate of *production* of fluid previously obtaining in the eye under normal conditions of circulation. The following table gives the rate of production of intraocular fluid, determined in this way, with varying intraocular pressures:—

| Animal. | Intraocular pressure in mm. Hg. | Inflow, after cessation of circulation, in cubic millimetres per minute. |
|---|---|---|
| Cat.................. | 20 | 12 |
| Cat.................. | 15 | 11 |
| Cat.................. | 26 | 12 |
| Cat.................. | 28 | 10 |
| Cat.................. | 14 | 5 |
| Cat.................. | 20 | 15 |
| Average...... | 20·5 | 10·8 |

It will be seen that there is a considerable difference in the case of filtration in various eyes, and therefore a corresponding difference in rate of production of intraocular fluid,

### IV. *The Factors Determining Absorption of Intraocular Fluid.*

In the last set of experiments we determined the rate of absorption of intraocular fluid at the normal intraocular pressure, and regarded this as representing the rate of production of this fluid under normal circumstances. In the same experiment it was possible to alter the intraocular pressure by raising or lowering the reservoir, and so to determine the effect of the height of the intraocular pressure on the rate of absorption. The results of two such experiments are given below, and show conclusively that the rate of absorption is determined, in the absence of disturbing factors which we shall have to consider later on, solely by the height of intraocular pressure.

(1) Cat, anæsthetised with Ether. While the anæsthesia was maintained, a small dose of morphia and curare was injected. Atropine was instilled locally into the conjunctival sac.

| B.P. in mm. Hg. | I.O.P. in mm. Hg. | Rate of inflow in cubic millimetres per minute. |
|---|---|---|
| 115 | 22 | 0 |
| 115 | 30 | 4 |
| 115 | 46 | 7 |
| 130 | 62 | 8 |
| | Heart divided. | |
| 0 | 22 | 12 |
| 0 | 36 | 16 |
| 0 | 46 | 19 |
| 0 | 62 | 22 |

(2) Cat, anæsthetised with Ether. Atropine and cocaine instilled locally into the conjunctival sac.

| B.P. in mm. Hg. | I.O.P. in mm. Hg. | Rate of inflow in cubic millimetres per minute. |
|---|---|---|
| 124 | 32 | 0 |
| 124 | 44 | 5 |
| 124 | 52 | 11 |
| 110 | 20 | 0 |
| 116 | 44 | 10 |
| 116 | 52 | 20 |
| | Heart divided. | |
| 0 | 52 | 22 |
| 0 | 44 | 15 |
| 0 | 20 | 12 |

In a previous paper we have shown that the intraocular pressure varies directly as the blood pressure in the vessels of the eyeball. We must therefore conclude that the rate of absorption of intraocular fluid is also determined by the height of the blood pressure, and since the absorption must keep pace exactly with the formation of this fluid, it follows that the formation of the intraocular fluid must also be determined by the height of the intraocular blood pressure. So far then the conditions which we laid down as necessary to be fulfilled in order to justify the filtration theory of the production of intraocular fluid have been fulfilled, and we might conclude with Leber that the formation of this fluid is exactly analogous to that of lymph, and is determined by the difference of pressure between the blood in the vessels and the fluid outside the vessels. There are, however, certain difficulties in this assumption which have so far not been considered by previous workers, but which must be met satisfactorily before we can come to any definite conclusion on the subject.

It has hitherto been assumed by Leber, Niesnamoff, and others, that a fluid having the composition of intraocular fluid might be formed by a process of filtration through the blood vessels of the ciliary processes under any difference of pressure. In this assumption they have neglected the question of the different proteid content of blood plasma and intraocular fluid. It was shown by one of us (E. H. S.) that, in order to separate a proteid-free transudate from a fluid such as blood serum, a certain amount of work had to be done, and that for this separation a minimum difference of pressure on the two sides of the filtering membrane of at least 28 mm. Hg was necessary. The intraocular fluid has such a small content in proteid that it may be regarded as analogous in all respects to the fluid which is supposed to be separated by the glomeruli of the kidney. In order therefore that any fluid shall be poured out in the eyeball, a minimum difference of 30 mm. Hg must be present between intraocular pressure and capillary blood pressure. If this pressure difference is not present, work must be done by the cells forming the filtering membrane, and the formation of intraocular fluid must be regarded in the light of a secretion rather than in that of a transudation. A definite decision on this point could be reached if we had any means of determining the blood pressure in the capillaries of the eyeball. A method for this purpose has been devised by Niesnamoff, (7) and this observer states that the normal intraocular capillary pressure is about 50 mm. of mercury. His arguments, however, involve several fallacies. In his experiments he connected a cannula, attached to a reservoir of salt solution, with the eyeball of a living animal. He found that the fluid neither ran in nor out at 25 mm. Hg, which was therefore the intraocular pressure. He then determined the rate of inflow when the pressure in his cannula was raised to 50 mm., 75 mm., and 100 mm. Hg. He then killed the animal, and again determined the rate at which the fluid would flow in under these various pressures. He found that above 50 mm. Hg the rate of inflow was the same in the dead as in the living animal. He therefore concluded that 50 mm. Hg represented the intracapillary pressure. In coming to this conclusion he was guided by the assumption that, when the intraocular pressure was raised so as to be equal to the intracapillary pressure, the transudation of intraocular fluid would cease, and above this pressure the rate of inflow for his reservoir would be, therefore, the same in the living and dead eye. It is impossible, however, by this method to determine intracapillary pressure. The globe of the eyeball is practically rigid. As the intraocular pressure is raised, the intraocular fluid will press upon the veins of the ciliary processes, and the blood pressure will therefore rise in the capillaries and in the veins until it is greater than the intraocular pressure. With successive rises in the intraocular pressure the pressure in capillaries and veins must get larger and larger in order that any circulation of blood may be maintained, and the circulation through the capillaries will cease only when the intraocular pressure is very nearly as high as the arterial pressure. If the circulation in Niesnamoff's experiments ceased at 50 mm. Hg, it is evident that the normal intracapillary pressure, when the intraocular pressure is 25 mm. Hg, must be considerably below 50 mm. Hg. How then are we to explain the very definite figures obtained by Niesnamoff? This observer apparently performed very few experiments. In his paper he gives the results of only one such experiment as that here described. On repeating his experiments we found it impossible to obtain anything like such definite figures—and this for various reasons. In the first place, a considerable rise of intraocular pressure, such as to 50 or 70 mm. Hg, exercises an abnormal stretching effect upon the filtering apparatus of the eyeball, so that the channels at the anterior angle of the eye are gradually opened up, and in many experiments we observed a consequent gradual increase in the rate of inflow of the fluid. In most experiments, for example, the rate of inflow was greater with descending pressures than with ascending pressures. This is well shown in experiment No. 2, on p. 301.

The following experiment shows the dilatation consequent on a preliminary raising of the intraocular pressure:—

Cat, anæsthetised with Ether. Eserine applied locally to conjunctival sac. Pupil moderately contracted.

| B.P. in mm. Hg. | I.O.P. in mm. Hg. | Rate of absorption in cubic millimetres per minute. |
|---|---|---|
| 110 | 16 | 0 |
| 110 | 32 | 5 |
| 110 | 48 | 8 |
| 108 | 64 | 9 |
| 112 | 16 | 0 |
| 112 | 32 | 8 |
| 112 | 48 | 13 |
| 112 | 64 | 18 |

Another disturbing factor is the size of the pupil. We shall have to consider this factor more in detail later on, but unless atropin be given at the beginning of the experiment, the observations on the living eye are made with a somewhat contracted pupil, whereas those on the dead eye are made on a widely dilated pupil. Other factors being equal, the filtration in the eye with dilated pupil is always slower than in the eye with contracted pupil. In certain of our experiments we observed an equality of inflow between the dead and living eye at some pressure above 40 mm. of mercury, but on further raising the pressure this equality disappeared, showing that we were dealing with yielding tissues and altering membranes. This fact rendered it impossible to obtain by such methods any definite information of the intracapillary pressure in the eye-ball, or of the level of intraocular pressure at which transudation or formation of intraocular fluid would definitely cease. One other factor which would aid in disturbing the results obtained is the effect of a high intraocular pressure on the general circulation through the eye-ball. If we succeed in raising the pressure to such a height that the circulation is entirely abolished, changes must rapidly take place in the apparatus both for formation and absorption of intraocular fluid, and subsequent results cannot be compared with those obtained before such a cessation of circulation. The raising of the intraocular pressure in itself may act as a stimulus and cause reflexly alterations in blood flow, in the general blood pressure, or in the state of contraction of the pupil. The co-operation of these various factors suffices to explain the varying results obtained in the very many experiments we performed upon this subject, including those of which we have already given details. We are of opinion, therefore, that the results obtained by Niesnamoff must be regarded as accidental, and that a greater number of experiments would have convinced this observer of the fallacies of his method.

Although it is impossible at present to determine the intracapillary pressure in the ciliary processes, we may at any rate inquire whether there is, in all experiments on the subject, the possibility of a difference of pressure of 30 mm. Hg between intracapillary blood pressure and intraocular pressure. In the case of a similar question in the kidney, we have been accustomed to compare the aortic blood pressure with the ureter pressure, and have regarded a difference of 40 mm. between these two pressures as satisfying the necessary conditions for filtration through the glomeruli. A similar comparison of arterial blood pressure and intraocular pressure leads to the same result. Below we give the intraocular pressure and arterial pressure as determined in a series of 20 experiments. It will be seen that in every case there is a difference between the two pressures of at least 48 mm. Hg, the average difference of pressure in all the experiments being 84·8 mm. Hg.

| Animal. | B.P. in mm. Hg. | I.O.P. in mm. Hg. | B.P. – I.O.P. |
|---|---|---|---|
| Cat................. | 130 | 16 | 114 |
| Cat................. | 140 | 25 | 115 |
| Cat................. | 138 | 20 | 118 |
| Cat................. | 94 | 24 | 70 |
| Rabbit ............ | 74 | 16 | 58 |
| Dog ........ ...... | 112 | 14 | 98 |
| Cat................. | 104 | 15 | 89 |
| Cat................. | 106 | 19 | 87 |
| Cat................. | 106 | 18 | 88 |
| Cat................. | 120 | 20 | 100 |
| Cat................. | 150 | 22 | 128 |
| Dog ............... | 84 | 12 | 72 |
| Dog ............... | 58 | 10 | 48 |
| Dog ............... | 70 | 16 | 54 |
| Cat................. | 115 | 23 | 92 |
| Cat................. | 124 | 32 | 92 |
| Cat................. | 110 | 16 | 94 |
| Cat................. | 138 | 22 | 116 |
| Cat................. | 94 | 27 | 67 |
| Cat................. | 110 | 24 | 96 |

So far then our observations tend to support in every particular the view laid down by Leber, namely, that intraocular fluid is produced in the ciliary processes by a process of filtration, and that the sole factor determining the amount of transuded fluid is the difference of pressure between the blood in the capillaries and the fluid in the eye-ball.

### V. *Influence of the Proteid Content of the Intraocular Fluid on the Intraocular Pressure.*

The fact that the intraocular fluid has to be filtered through the intercellular channels of the endothelium bounding the spaces of Fontana and lining the canal of Schlemm, in order to escape from the eyeball, suggests that the resistance will be greater if the viscosity of the filtering fluid be increased in consequence of raised proteid content. Indeed, one form of raised intraocular pressure, the glaucoma accompanying inflammation of the ciliary region, has been ascribed to the greater proteid content of the intraocular fluid secreted by the inflamed vessels, and the consequent greater resistance to the filtration of this fluid through the anterior angle of the eye. So far as we are aware, there are no direct determinations of the relative rates of filtration of normal salt solutions with and without proteid. We have, therefore, in a series of animals, determined the intraocular pressure under the two conditions:—

(*a*) With normal intraocular fluid.
(*b*) After replacing this fluid by blood serum.

We have also compared the relative rates of filtration of normal salt solution and of serum in the living and dead eye.

In our experiments one eye of the animal was connected with a reservoir and manometer containing Ringer's saline fluid, while the other was connected with a similar apparatus filled with filtered blood serum.

In order to determine the intraocular pressure in an eye, in which the normal aqueous humour had been replaced by serum, after introduction of the hollow needle, the aqueous was allowed to escape through the side opening in the cannula. Serum was then allowed to flow in for a time, and then the contents of the anterior chamber again allowed to escape. The side tube was then closed, an air bubble introduced into the capillary tube, and the pressure determined at which the bubble moved neither backwards nor forwards.

In nearly every experiment the intraocular pressure, during the first 5 or 10 minutes after the insertion of the cannula, was higher in the eye filled with serum than in the eye filled with normal fluid. The difference, however, rapidly diminished, so that 15 to 20 minutes after the beginning of the observation the pressures were practically identical in the two eyes, and remained so throughout the rest of the experiment. It must be remembered that with the zero method used by us there is no movement of fluid into the eye. Hence the fluid necessary to replace the loss by filtration and to maintain the intraocular pressure is being constantly secreted by the ciliary processes, and is probably of the normal composition, *i.e.*, practically free from proteid. We should therefore expect a gradual decline of the intraocular pressure in the eye with serum, although hardly so rapid an equalisation of the pressures on the two sides as we actually observed in our experiments.

After the determination of the intraocular pressure, the animal was killed by opening its heart, and the inflow of serum and saline fluid respectively observed, first under the normal intraocular pressure, and then under raised pressures.

The results of two such experiments are given below. It will be seen that there is a marked difference in the rate of filtration of the two fluids, that of serum being, as one might predict, very much slower than that of saline.*

Experiment 1.—Dog, A.C.E. Morphia. Curare. Vagi cut.

| Time. | Blood pressure. | Intraocular pressure. | |
|---|---|---|---|
| | | Salt eye. | Serum eye. |
| 4.15 | 70 mm. Hg. | 26·2 | 29·4 cm. water. |
| 4.20 | 70 „ | 24·2 | 27 „ |
| 4.45 | 100 „ | 29·2 | 29 „ |
| Animal killed | by opening heart. | | |

| Pressure. | Inflow per minute in cubic millimetres (after 10 minutes). | |
|---|---|---|
| | Salt. | Serum. |
| 29 cm. | 11·5 | 6 |
| — | 11·5 | 6 |
| — | 11·5 | 6 |

Experiment 2.—Cat. Ether, morphia, curare.

| Time. | Blood pressure. | Intraocular pressure. | |
|---|---|---|---|
| | | Salt. | Serum. |
| 3.0 P.M. | 120 | 14·8 | 15·1 |
| 3.10 | 116 | 10·8 | 12·5 |
| 3.20 | 110 | 9·2 | 11·5 |
| Animal killed. | | | |

---

* Although serum filters more slowly than normal intraocular fluid or saline, the difference is not sufficiently great to cause any marked variation in the intraocular pressures on the two sides. One cannot, therefore, in view of these observations, ascribe any large part in the production of any form of glaucoma to possible differences in the composition of the aqueous humour which might be determined by inflammatory conditions of the blood vessels.

Inflow three minutes later at same intraocular pressures—

| Salt. | Serum. |
|---|---|
| 6 | 3 |
| 5 | 3·5 |
| 5 | 6 |
| 5 | 4 |
| 4 | 4 |

Fifteen minutes later—

| | |
|---|---|
| 3·5 | 1·5 |
| 3·0 | 1·5 |
| 3·5 | 1·5 |
| etc. | etc. |

This difference in the rate of filtration of the two fluids becomes greater the higher the intraocular pressure is raised.

## VI. *The Effect of the Size of the Pupil on the Absorption of Intraocular Fluid.*

In the experiments we made to decide this point, one eye of the animal under observation was treated with eserine and the other with atropine. The instillation of these drugs should be begun before the induction of anæsthesia, as the action of eserine is very uncertain if only instilled after anæsthesia.

We have found, as a result of these experiments, that the intraocular pressure in the two eyes remains the same during the time of observation, but that, if the pressure in the apparatus be raised, the rate of filtration in the eye under eserine is much greater than in that under atropine.

It is difficult to give a precise explanation as to the cause of this difference. Stretching of the filtration spaces at the angle of the anterior chamber may possibly account for it all. If this, however, is the case, we should expect to find the intraocular pressure at a lower level in the eye with the contracted pupil, for the intraocular pressure must of course be the product of the rate of secretion and the rate of absorption of the intraocular fluid. The same objection applies to the explanation of this phenomenon by Grönholm (9), who states that in his opinion it is due to diminished intraocular secretion as a result of the contraction of the intraocular vessels. It may also be possible that at these raised pressures other channels of filtration are opened up—such for instance as the surface of the iris. An important, perhaps the most important, factor, however, must be the crushing of the dilated flaccid iris into the filtration angle of the eye, thus causing a mechanical obstruction, which will be more marked the greater the intraocular pressure. Hence the smaller amount of filtration in the atropinised or dead eye with dilated pupil, as compared with that in the eye which has been put under the influence of eserine.

The figures of a typical experiment are given.

Cat, anæsthetised with Ether. Blood pressure average 138 mm. Hg, with only trifling variations throughout the experiment.

| Intraocular pressure in mm. Hg. | Rate of filtration in eserine eye in cubic millimetres per minute. | Rate of filtration in atropine eye in cubic millimetres per minute. | Rate of filtration in atropine eye *post-mortem*, in cubic millimetres per minute. |
|---|---|---|---|
| 20 | 0 | 0 | 15 |
| 35 | 11 | 8 | 20 |
| 50 | 16 | 11 | 25 |
| 65 | 23 | 14 | 31 |

### *Summary of Conclusions.*

1. The intraocular pressure represents the pressure at which the rate of formation of intraocular fluid is exactly balanced by its rate of escape through the filtration angle of the eye.

2. The production of intraocular fluid is strictly proportional to the difference of pressure between the blood in the capillaries of the eyeball and the intraocular fluid.

3. No satisfactory method of measuring the intracapillary pressure in the eyeball has been yet devised. The fallacies of Niesnamoff's method are pointed out. Judging, however, from a comparison of the arterial pressures and the intraocular pressures in a large number of animals under different conditions, there is probably always a difference between the intracapillary pressure and intraocular pressure, which is sufficient to account for the production of the intraocular fluid, without assuming any active intervention on the part of the cells of the capillary walls or of the ciliary processes.

4. An increased proteid content of intraocular fluid slows its rate of absorption in consequence of the mechanical hindrance of the proteid to filtration.

5. Filtration, *i.e.*, the absorption of intraocular fluids, at high intraocular pressures is favoured by constriction of the pupil and hindered by dilatation of the pupil. The difference, however, is barely perceptible with normal or low intraocular pressures.

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# *On the Filtration of Crystalloids and Colloids through Gelatine: with special reference to the behaviour of Hæmolysins.*

By J. A. Craw, British Medical Association Research Scholar.

(Communicated by Leonard Hill, M.B., F.R.S. Received December 1, 1905,—
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The current controversy between Ehrlich (1898, 1903) and Arrhenius and Madsen (1902, 1904) on the physical chemistry of the neutralisation of toxins by their specific antitoxins led the author (IV, 1905) to an investigation of the relations existing between the toxin for red blood corpuscles secreted by *B. megatherium* and its specific antiserum. One of the methods adopted consisted in the filtration of mixtures of megatherium hæmolysin and antilysin through gelatine. The lysin was found to pass into and even through the gelatine, whereas the antilysin was retained, and by means of the delicate blood test for free lysin it was possible to demonstrate that the two substances, on mixing in any proportions, do not completely neutralise each other. These observations indicated that Ehrlich's views on the toxin-antitoxin reaction required considerable modification, but a closer investigation showed that the hypcthesis advanced by Arrhenius and Madsen agreed even less with the experimental facts. On the other hand, the results were in entire harmony with the views advanced by Landsteiner (1903) and Bordet (1903), which have been supported by Nernst (1904) and Craw (1905) that the toxin is adsorbed by the antitoxin much as a dye is by a tissue. As this conclusion may considerably modify current ideas on the nature of the reaction and the constitution of toxins in general, it seemed advisable to inquire further into the physical chemistry of filtration through gelatine.

The present communication contains data of the filtration of various crystalloidal and colloidal solutions, including megatherium lysin, through various percentages of gelatine, under constant and variable pressures.

The work was partly carried out as Research Student at the Lister Institute of Preventive Medicine and was completed at the London Hospital Medical College by the aid of a scholarship from the British Medical Association.

*Previous Work on the Gelatine Filter.*—The gelatine filter was introduced by C. J. Martin (1896), and consists of a Pasteur-Chamberland candle, the pores of which are filled with solid gelatine. The filter is fitted into a gunmetal jacket or filter case, which serves to hold the liquid to be filtered, and the upper end of the closed filter case is connected with a supply of air at a pressure of 30 to 100 atmospheres, which is used to force the liquid through the gelatine. From his observations with "wet" gelatine filters, *i.e.*, filters containing gelatine from which the normal content of water had not been removed by drying in air or otherwise, Martin* concluded that gelatine was impermeable to colloidal substances such as globulin, albumin, glycogen, and soluble starch, but partially permeable to albumoses and dextrin, and completely permeable to solutions of crystalloids, *e.g.*, urea and dextrose. It seemed, then, as if the gelatine filter was an instrument destined to play an important part in the investigation of physiological fluids.

Martin and Cherry (1898) applied the filter to the investigation of the course of the reaction occurring between diphtheria toxin and antitoxin, and likewise to the reaction of snake venom with antivenene, the toxin and venom being filter-passers, whereas the anti-bodies† were retained.

From these experiments it seemed as if the toxin was completely neutralised by the antitoxin, but further investigation of the mechanism of gelatine filtration shows that no such absolute conclusion can be drawn. E. Waymouth Reid (1901) showed conclusively that crystalloids do not pass "wet" gelatine filters in unaltered concentration, and that although filters which had been dried to constant weight in dry air allowed certain crystalloidal solutions to pass unchanged, yet dextrose and sodium oleate‡ were partially retained. Further, he found that the filtrate from serum§ had not the same composition as the proteid-free serum, and that the residual fluid left in the filter case had a much higher concentration of organic substances of non-proteid character than either the original serum or the filtrate. The Martin filter is not, therefore, a simple means of separating crystalloids from colloids. One must not, however, under-estimate its value as an instrument for the analysis of physiological fluids, for although the filter shows considerable differences in permeability to various crystalloidal substances, I find (IV, 1905) that these and inferior colloids are, on the whole, retained to a small extent compared with typical colloids. The partial retention of filter-passers has an important bearing on the conclusions to be drawn from gelatine filtrations, for, if the concentration of the filter-passer to be tested for be small in the original fluid introduced into the filter, the gelatine may retain practically the whole amount and the filtrate contain only a quantity below the experimental error of observation. This was found to be the case for neutral mixtures of megatherium lysin and antilysin, and mixtures containing excess of antilysin, by the author;* the filtrates showed no hæmolytic power, whereas the gelatine had stored up a considerable amount of free megatherium lysin. In this light Martin and Cherry's observations on diphtheria toxin and snake venom, mentioned above, are in entire agreement with my results for megatherium lysin, the free toxin and venom of the neutral mixtures being probably stored up in the gelatine of their filters.

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* *Loc. cit.*

† *Cf.* also Brodie (1897) (1900).

‡ Krafft (1902) considers soap solutions, such as sodium oleate, to be colloidal.

§ *Cf.* also Starling (1899).

*On "Wet" and "Dry" Gelatine Filters.*—Martin and others used the filters in the "wet" state, *i.e.*, shortly after solidification of the gelatine in the pores of the candle, and, therefore, containing a considerable quantity of water, part of which on filtration will pass into the filtrate and so dilute the substance filtered. To get rid of this difficulty, E. W. Reid removed part of the water by drying Martin candles to constant weight in a current of dry air. It seemed to me, however, that by drying the gelatine another difficulty might be introduced, viz., a change in the size of the pores, which would render observations with "dry" filters of doubtful value.

*Method.*—The rate of filtration of water through freshly prepared wet filters was observed during one hour and compared with the rates during a similar period, of filters which had been partially dried by standing in dry air for 10 hours, 24 hours, and 3 days.

*Rates of Filtration.*—The freshly prepared wet filters gave fairly uniform rates, and allowed from 0·5 to 2 c.c. of water to pass per minute at 100 atmospheres pressure, the concentration of the gelatine in the pores being 9 per cent. and the temperature 10° to 15° C. Under the same conditions the partially dried filters allowed from 5 to 20 c.c. to pass per minute at the beginning of the filtration, but the rate rapidly decreased. On reducing the applied pressure to about one atmosphere, and allowing the water to flow through a partially dried filter at the rate of about 1 to 2 c.c. per minute, the filter gradually tightened, so much that after the passage of 30 to 50 c.c. a pressure of 100 atmospheres was necessary to maintain the same rate. The longer the filters were dried the more marked was the porosity and the greater the amount of fluid which had to be pressed through before the filter tightened.

*Conclusions.*—On partially drying gelatine filters the gelatine shrinks, and air passages are produced of greater diameter than the water passages of a wet filter. The wide dry filter passages at first offer a free passage to the fluid filtered, and no material change is to be expected in the percentage composition of the latter. The filter gradually changes in character until ultimately, a wet filter is obtained with pores of similar dimensions to those of a freshly prepared wet filter. The various fractions of filtrate from a

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* *Loc. cit.*

"dried" filter are not subject to the same conditions of filtration and are not, therefore, comparable. Wet filters, on the other hand, show a much greater constancy in rate of filtration, and are, therefore, to be preferred.

*On the Preparation of the Filters.*—To overcome the difficulty of dilution of the filtrates by the water of wet filters, the gelatine to be used in filling the candles was in several cases dissolved in the fluid to be filtered. Thus the filtration of 0·8 per cent. sodium chloride and of megatherium lysin took place through 9 per cent. gelatine which had been dissolved in 0·8 per cent. sodium chloride, and 1·54 per cent. potassium iodide was filtered through 11 per cent. gelatine in 1·54 per cent. iodide. A Pasteur-Chamberland candle of size B, which had in the majority of cases been heated for 10 hours at least in a muffle furnace to remove organic matter, was fixed into a brass socket with "Faraday" cement and fitted concentrically into the internally tinned gunmetal jacket or "filter case." After thoroughly washing through with about 400 c.c. of hot water and 250 c.c. of the solution of French gelatine to be used in forming the filter, at a temperature of 37° C., the gelatine at 30° C. was slowly passed through the cooling filter at an air pressure just sufficient to cause about 1·5 c.c. or 15 drops to filter per minute. The filtration was continued until no trace of air bubbles was visible in the drops, after which a further 50 c.c. were allowed to pass. During the final filtration the upper surface of the gelatine solution in the filter case was not allowed to descend below a level 3 cm. above the crown of the candle. The filter was then removed, drained, placed in the neck of a flask containing a little water, and kept in the ice chest at 6° to 8° C. until required. If the filter so prepared had an obviously thick skin of gelatine upon its surface, this was removed by immersing in the gelatine solution at 30° C. and draining as before. In the comparative experiments with different concentrations of gelatine, the filters were treated in the manner last described to get membranes of approximately equal thickness. Only those filters were used which allowed less than 2 c.c. or about 20 drops per minute to pass at 100 atmospheres pressure and 10° to 15° C., and the majority of filters allowed only about 0·5 c.c. per minute to pass under these conditions. After use the candles could be washed out with water at 50° C., dried, heated in a muffle furnace, and on refilling with gelatine again gave reliable filters.

### *Filtration of a Typical Crystalloid, Sodium Chloride, through 9 per cent. Gelatine.*

Sodium chloride was chosen on account of its characteristic crystalloidal properties and the important *rôle* it plays in physiological fluids. Further, in a previous communication,* it was found that the filtrates from megatherium lysin were strongly hæmolytic, and it was necessary to determine if this hæmolysis was due in any degree to change in the concentration of sodium chloride.

*Method.*—The filters used contained 9 per cent. gelatine in 0·8 per cent. NaCl, and about 50 c.c. of 0·8 per cent. NaCl were pressed through at 100 atmospheres and 10° to 12° C., the filtrates being collected in fractions of about 4 c.c. The NaCl content of 1 c.c. of the fractions of filtrate, the residual liquid left in the filter case and the original fluid, was determined by titration with 1/100 normal silver nitrate, using potassium chromate as an indicator. The hæmolytic powers of the same fluids were determined in these and all other experiments in this paper, unless otherwise stated, by mixing 1 c.c. of the respective fractions with 2 c.c. of 2·5 per cent. guinea-pig's red corpuscles which had been washed and suspended in 0·8 per cent. NaCl. The mixtures were heated to 37° C. for three hours, well shaken every 30 minutes, and allowed to sediment generally about 12 hours in the ice chest at 6° to 8° C.

The intensity of tint of the supernatant fluid was then determined by comparison with the scale of a von Fleischl hæmoglobinometer which had been standardised by a blood solution of known content. Complete hæmolysis is indicated by the index (100).

*Examination for Sodium Chloride and Hæmolytic Power.*—Experiment No. 2, Table I, represents one out of four similar experiments.

Original Fluid: 0·81 per cent. NaCl given in the table as Orig. (100).

Filtrates: The relative concentrations of the 1st, 4th, and 14th fractions were (47·1), (89·5), and (99·2) respectively.

Residual Fluid: When tested immediately after decompression had the value (100) exactly, but, on allowing to stand 12 hours in the filter case, showed the relative concentration (105).

Hæmolytic Powers: No hæmolysis was obtained under the standard conditions with either filtrates, residual fluid, or the original saline.

*Conclusions.*—The typical crystalloid, sodium chloride, is markedly retained on filtration through gelatine which originally contained the same concentration of saline as the fluid filtered. The salt taken up by the gelatine is expressed or diffuses into the residual fluid after decompression. The diminution in salt concentration in the filtrate is insufficient to cause hæmolysis under the standard conditions.

* *Loc. cit.*

### *Continued Filtration of Sodium Chloride through* 9 *per cent. Gelatine.*

As the last fraction of filtrate in No. 2 did not quite reach (100), it seemed desirable to ascertain whether on continued filtration this value could be obtained.

*Method.*—The residual fluid of No. 2, which had stood in the filter case overnight, was poured out, and the filter case refilled with 112 c.c. of saline, of which 40 c.c. in 5 c.c. fractions were pressed through.

Table I.

| | Sodium chloride, per cent. | | | | | | Megatherium lysin, H.Is. | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| No. ... | 1. | 2. | 2A. | 2B. | 3. | | 4. | 5. | 6. | 7. | 8. |
| P. c. ... | 0·88. | 0·81. | | | | HBū. | 15. | 11. | 9. | 7·5. | S. |
| Successive fractions of filtrate. | 23·9 | 47·1 | 101·5 | 97·4 | 16·7 | 0·2 | 0·7 | 0·5 | 0·0 | 0·5 | 0·0 |
| | 31·4 | 55·1 | 103·3 | 97·8 | 41·7 | 5·8 | 0·5 | 0·0 | 0·0 | 5·0 | 3·5 |
| | 39·0 | 76·1 | 99·6 | 96·0 | 47·8 | 60·4 | 0·0 | 2·3 | 0·0 | 17·7 | 10·7 |
| | 49·0 | 89·5 | 101·1 | 93·1 | 56·9 | 74·0 | 0·0 | 5·1 | 3·7 | 25·0 | 17·8 |
| | 56·0 | 95·3 | 99·6 | 94·2 | 65·6 | 85·4 | 0·0 | 5·1 | 27·8 | 34·6 | 21·4 |
| | 64·1 | 96·7 | 97·6 | 93·5 | 70·6 | 88·5 | 0·0 | 7·1 | 46·3 | 45·1 | 25·0 |
| | 71·1 | 97·6 | 100·7 | 95·7 | 76·8 | 88·5 | 0·0 | 8·6 | 33·9 | 48·8 | 28·5 |
| | 76·1 | 97·6 | 97·6 | 94·2 | 80·4 | 90·6 | 0·0 | 8·6 | 47·8 | 48·8 | 38·5 |
| | 79·2 | 97·6 | — | 94·2 | 83·1 | 90·6 | 0·0 | 8·6 | 47·0 | 48·8 | 32·1 |
| | 81·7 | 99·2 | — | 96·0 | 85·0 | 90·6 | 0·0 | 12·3 | 47·0 | 48·4 | 32·1 |
| | 83·3 | 99·6 | — | 94·9 | 86·3 | 90·6 | 0·0 | 12·8 | 45·6 | 48·4 | 35·7 |
| | 112·6 | 100·0 | — | 96·8 | — | — | 2·3 | 8·0 | 47·0 | 49·3 | 35·7 |
| | 107·8 | 99·6 | — | 96·4 | — | — | 3·7 | 10·0 | 46·3 | 48·8 | 50·0 |
| | 107·8 | 99·2 | — | 96·4 | — | — | 10·0 | — | — | 48·4 | 50·0 |
| | 98·4 | — | — | 96·4 | — | — | — | — | — | — | 50·0 |
| | 96·2 | — | — | — | — | — | — | — | — | — | 50·0 |
| | 93·7 | | | | | | | | | | |
| Res. | 134·9 | 100·0 | 100·0 | 100·0 | 110·0 | 125·0 | 85·0 | 62·9 | 55·6 | 52·7 | 67·8 |
| | c.c.<br>20 | c.c.<br>44 | c.c.<br>65 | c.c.<br>40 | c.c.<br>45 | — | c.c.<br>47 | c.c.<br>45 | c.c.<br>34 | c.c.<br>46 | c.c.<br>31 |
| Orig. | 100·0 | 100·0 | 100·0 | 100·0 | 100·0 | 100·0 | 50·0 | 50·0 | 50·0 | 50·0 | 50·0 |
| | c.c.<br>100 | c.c.<br>112 | c.c.<br>112 | c.c.<br>120 | c.c.<br>105 | — | c.c.<br>110 | c.c.<br>105 | c.c.<br>100 | c.c.<br>110 | c.c.<br>105 |

*Examination for Sodium Chloride.*—Experiment No. 2A, Table I.

Original Fluid: 0·81 per cent. NaCl = Orig. (100).

Filtrates: The 1st and 2nd fractions contained (101·5) and (103·3) respectively, and those succeeding showed values varying about (100).

Residual Fluid : Titrated immediately after decompression (100), after 12 hours in the filter case (110).

*Conclusion.*—On renewing filtration the filtrate may contain a higher percentage of salt than the original fluid, due probably to the temporary decompression, for on continued recompression the concentration tends to become less than (100).

### *Continued Filtration of Sodium Chloride with Megatherium Lysin and Antilysin through* 9 *per cent. Gelatine.*

In a former paper,* mixtures of lysin and antilysin were pressed through gelatine which had been tested for tightness by the filtration of saline. The redistribution of salt under these circumstances was now determined.

*Method.*—A filter through which 10 c.c. of 0·81 per cent. NaCl had been pressed, the original gelatine being 9 per cent. in 0·81 per cent. NaCl, was decompressed, the residual salt solution removed, and the filter case refilled with 120 c.c. of a nearly neutral mixture of equal volumes of a fluid megatherium lysin and 5 per cent. antilysin in saline, of which 75 cc. in 5 c.c. fractions were pressed through.

*Examination for Sodium Chloride.*—Experiment No. 2B, Table I, is one of two practically identical filtrations.

Original Fluid: Contained 0·81 per cent. NaCl = (100). Hæmolytic Index (16·8).

Filtrates: As in No. 2A the 1st and 2nd fractions were the most concentrated in NaCl, but they did not reach (100), and the final fraction was (96·4). The average hæmolytic index of the whole filtrate was (0·5).

Residual Fluid: The results were entirely similar to those of No. 2A as regards NaCl. Hæmolytic Index (38·6).

*Conclusion.*—The redistribution of salt is insufficient to give a trace of hæmolysis under standard conditions.

Temporary decompression even for a few minutes allows a higher percentage of salt to pass into the filtrate than would be obtained by constant pressure.

### *Filtration of Sodium Chloride with Butyric Acid through* 9 *per cent. Gelatine.*

Butyric acid in saline acts as a very strong hæmolysin, and, as it possesses crystalloidal properties, it appeared to be of interest to compare its filtration phenomena with those of megatherium lysin in saline. Further, butyric acid greatly diminishes the surface tension of saline against air, and the possibility of a change in the surface forces between saline and gelatine formed a second point of interest, which is discussed towards the end of this paper.

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* *Loc. cit.*

*Method.*—A solution of 0·81 per cent. NaCl containing 4·8 per cent. butyric acid was pressed through 9 per cent. gelatine in 0·81 per cent. NaCl at 100 atmospheres. The content of the fractions of filtrate, etc., in NaCl was determined as before, and the butyric acid was estimated by titration with standard sodium hydrate, using phenol-phthalein as indicator. The hæmolytic powers of the various fluids were also determined by the time taken for 1 c.c. of the fractions to hæmolyse completely 2 c.c. of a 2·5 per cent. suspension of guinea-pig's corpuscles in 0·8 per cent. NaCl at 16° C.

*Examination for Sodium Chloride.*—Experiment No. 3, Table I. The redistribution of salt was qualitatively similar to that which had been obtained in the absence of butyric acid. The amount passing into the filtrate was, however, smaller.

*Examination for Butyric Acid and Hæmolytic Power.*—Original: 4·8 per cent. butyric acid = (100) HBū. Hæmolytic time = 8 minutes.

Filtrates: The 1st and 2nd fractions contained (0·2) and (5·8) HBū respectively. The 1st fraction agglutinated the test blood strongly, and the 2nd hæmolysed completely in 50 minutes. The 8th to the 11th fractions contained (90·6) HBū, and the hæmolytic time was 12 minutes.

Residual Fluid: Concentration (125) HBū. Hæmolytic time, 5 minutes.

*Conclusions.*—Butyric acid is retained by gelatine to a considerable extent. The gelatine appears to retain more sodium chloride in the presence of butyric acid.

### *Filtration of Sodium Chloride through* 9 *per cent. Formalised Gelatine.*

It seemed probable, from the marked change which takes place in gelatine on being exposed to formic aldehyde, that such gelatine would show a different permeability to that already found for ordinary gelatine. On the other hand, the possibility of investigating physiological fluids in an apparatus which could be thoroughly and easily sterilised by means of "formalin" might recommend the use of formalised gelatine filters.

*Method.*—A filter containing 9 per cent. gelatine in 0·8 per cent. NaCl was fitted into the filter case, and the latter filled with a solution of 0·8 per cent NaCl containing 10 per cent. of commercial "Formalin." A few cubic centimetres of fluid were pressed through, and the remainder allowed to stand in the filter case overnight. The solution was then removed, and, after about 12 hours, 100 c.c. of 0·88 per cent. NaCl were placed in the filter case, and pressed through in fractions of 4 to 5 c.c.

*Examination for Sodium Chloride.*—Experiment No. 1, Table I.

Original Fluid: 0·88 per cent. NaCl represented as Orig. (100).

Filtrates: No formaldehyde or formic acid could be detected. From the 1st to the 11th fraction the NaCl gradually increased from (23·9) to (83·3), at 100 atmospheres filtration pressure. At this point the pressure was removed and filtration resumed after an interval of eight hours. Six fractions of 5 c.c. each were then removed, and gave values decreasing from (112·6) to (93·7).

Residual Fluid: Tested immediately after the last decompression contained (134·9).

*Conclusions.*—Formalised gelatine containing sodium chloride retains sodium chloride from a solution, on filtration, to a greater extent than ordinary gelatine. The decompression in this case also leads to the immediately following filtrates having a higher concentration of salt than the original fluid, but the effect is more marked than with ordinary gelatine. The concentration of the residual fluid is also much greater than with ordinary gelatine.

### *Filtration of Megatherium Lysin Through Various Percentages of Gelatine.*

Megatherium lysin diffuses slowly compared with crystalloids in general,* and is probably of semi-colloidal character. It seemed probable that a substance of this type would be considerably more affected by a change in the concentration of gelatine than a good filter-passer such as sodium chloride. Hardy's (1899) work on gelatine pointed also to the possibility of considerable change in the structure of the jelly at about 7 per cent. gelatine, which might lead to markedly different degrees of permeability between 7 and 15 per cent gelatine filters.

*Method.*—Filters containing 15, 11, 9 and 7·5 per cent. gelatine in 0·8 per cent. NaCl were tested for tightness with 0·8 per cent. NaCl, of which 10 c.c. were pressed through in each case to ensure a concentration of NaCl in the succeeding filtrates of about 0·8 per cent. The fluid examined was the hæmolytic filtrate from a broth culture of *B. megatherium* diluted with an equal volume of 0·8 per cent. NaCl.

Filtration was carried through at a pressure of 100 atmospheres and a temperature of 10° to 12° C. and the filtrate was collected in fractions of about 4 c.c.

*Examination for Lysin.*—Table I.

Original Fluid.—The hæmolytic index (see above) was (50).

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* *Cf.* Craw (IV, 1905).

Filtrates: Experiments Nos. 4, 5, 6 and 7, Table I, show the hæmolytic values of the succeeding fractions through 15, 11, 9 and 7·5 per cent. gelatine respectively. The average indices for the whole filtrates were in the order given above (1·5), (6·9), (31·7), and (36·9).

In Experiment No. 8, Table I, 50 c.c. of 20 per cent. horse serum in 0·8 per cent. NaCl had previously been passed through a 7·5 per cent. gelatine filter. In this case the permeability to lysin was slightly less than that of a similar filter without serum, the average indices of the filtrates being (30·6) and (36·9) respectively.

Gelatine: On melting out the gelatine, after filtration, at 37° C., and mixing with the test blood, the latter was in all cases rapidly and completely hæmolysed, index (100). Control experiments showed that saline on being pressed through gelatine which had been used to filter lysin became strongly hæmolytic. The original gelatine had no hæmolytic effect in the standard time.

Residual Fluids: The residual fluids in all cases showed average indices which were higher than that of the original fluid. The lowest portions of residual fluid had generally higher indices than the portions towards the upper surface. With rising concentration of gelatine the hæmolytic power increased, thus with 7·5 per cent. the residue had an index (52·7) whereas with 15 per cent. it was (85).

*Conclusions.*—Megatherium lysin is retained to a greater extent than sodium chloride, and more is retained with higher concentrations of gelatine. The residual fluids have higher concentrations than the original and the concentration is greater with higher percentages of gelatine. Diffusion and expression of lysin from the gelatine into the residual fluid are insufficient to account for the increased concentration immediately after decompression, and it seems as if the water of the original fluid could pass into the gelatine more readily than the lysin.

It seems possible, under the conditions of preparation, that during the draining of the filters from the various percentages of gelatine at 30° C., the filters with the higher concentrations would retain thicker surface layers of gelatine and so exaggerate the differences in permeability.

### *Filtration of a Typical Colloid, Ferric Hydrate, through* 11 *per cent. Gelatine under Varying Pressure.*

As ferric hydrate in colloidal solution shows no appreciable tendency to diffuse, any redistribution of the colloid by the gelatine filter must be explained on some other basis than that of diffusion.

*Method.*—A 5-per-cent. solution of colloidal ferric hydrate was prepared by adding ammonium carbonate to ferric chloride and dialysing for three weeks; it gave no trace of red coloration with potassium thiocyanate, but showed the characteristic reaction of colloidal ferric hydrate, viz., a slight yellowish precipitate. The filter used contained 11 per cent. gelatine which had been dialysed for 24 hours. The content of the fractions of filtrate, etc., in iron, was determined by converting the hydrate into chloride and estimating colorimetrically with potassium thiocyanate.

Table II.

| | Colloidal ferric hydrate. | | | Neutral red. | | | Iodine in potassium iodide. | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Vol. in c.c. | Pressure in atmos. | Percentage Fe. | Vol. in c.c. | Pressure in atmos. | Percentage N.R. | Vol. in c.c. | Pressure in atmos. | Percentage $I_2$. | Percentage KI. |
| Successive fractions of filtrate. | 4 | 100 | 0·0 | 4 | 100 | 0·0 | 1 | 100 | 5 | 68·8 |
| | 4 | 100 | 0·7 | 4 | 100 | 2·3 | 4 | 100 | 8 | 69·9 |
| | 4 | 100 | 0·6 | 4 | 100 | 0·04 | 4 | 100 | 17 | 70·9 |
| | 4 | 100 | 0·4 | 3 | 20 | 0·03 | 4 | 100 | 19 | 69·9 |
| | 4 | 100 | 0·4 | 10 | 100 | 0·30 | 4 | 100 | 21 | 69·9 |
| | 4 | 100 | 0·4 | 10 | 100 | 0·78 | 4 | 100 | 23 | 69·9 |
| | 4 | 100 | 0·1 | 10 | 100 | 0·90 | 4 | 100 | 25 | 68·3 |
| | 4 | 100 | 0·0 | 10 | 100 | 1·17 | 1 | 15 | 20 | 69·9 |
| | 4 | 100 | 0·0 | 10 | 100 | 3·00 | 5 | 100 | 33 | 71·5 |
| | 1 | 100 | 0·0 | 10 | 100 | 3·20 | 5 | 100 | 33 | 73·0 |
| | 0·5 | 20 | 250·0 | 20 | 100 | 4·68 | 4 | 100 | 35 | 72·0 |
| | 1 | 100 | 4·75 | — | — | — | 0·75 | 0 | 0 | 43·0 |
| | 2 | 100 | 0·9 | — | — | — | 0·75 | 100 | 25 | 93·5 |
| | 3 | 50 | 0·7 | — | — | — | 6 | 100 | 37 | 90·5 |
| | 0·5 | 15 | 66·6 | — | — | — | 6 | 100 | 50 | 86·0 |
| | 0·75 | 50 | 1·0 | — | — | — | 1 | 0 | 1 | 34·4 |
| | 0·25 | 30 | 0·6 | — | — | — | 5 | 10 | 25 | 94·6 |
| | 0·5 | 20 | 0·3 | — | — | — | 2 | 100 | 30 | 92·5 |
| | 0·75 | 10 | 0·1 | — | — | — | 3 | 50 | 30 | 96·8 |
| | 0·25 | 50 | 0·2 | — | — | — | 4 | 100 | 30 | 91·4 |
| | 1 | 100 | 0·2 | — | — | — | 5 | 50 | 30 | 85·0 |
| | 5 | 15 | 0·4 | — | — | — | 5 | 25 | 30 | 82·8 |
| | 4 | 100 | 0·4 | — | — | — | 5 | 25 | 25 | 79·5 |
| | 0·5 | 15 | 0·4 | — | — | — | 5 | 25 | 25 | 73·1 |
| | 4 | 100 | 0·4 | | | | | | | |
| Res. | 50 | — | 180·0 | 20 | — | 18·75 | 35 | — | 30 | 109·7 |
| Orig. | 120 | — | 100·0 | 120 | — | 100·0 | 120 | — | 100 | 100·0 |

*Examination for Ferric Hydrate.*—Table II gives the relations obtained in one out of three similar experiments.

Original Fluid: Five per cent. ferric hydrate = (100) Fe.

Filtrates: The 1st and 2nd fractions contained (0·0) and (0·7) respectively, and in the succeeding fractions the amount diminished until in the 8th no trace could be found at 100 atmospheres filtration pressure. On suddenly diminishing the pressure to 20 atmospheres the liquid percolating through was very intensely coloured and gave the value (250). Re-establishing the pressure of 100 atmospheres, the filtrates became less and less intensely coloured (4·75) and (0·9). A drop in the pressure to 50 atmospheres did not materially change the concentration, but a further drop to 15 atmospheres gave a filtrate with the value (66·6). Increasing the pressure to 50 atmospheres caused the filter to tighten once more with respect to the hydrate, and on gradually decreasing the pressure to 10 atmospheres the filter remained tight. In this condition sudden variations of pressure from 100 to 15 atmospheres had but a slight effect on the permeability of the filter.

Residual Fluid: The colour was more intense than that of the original fluid and its content was (180) Fe.

*Conclusions.*—The gelatine fifter is slightly permeable to the typical colloid ferric hydrate, but at constant pressure the permeability decreases as the filtration proceeds. The permeability is increased enormously by suddenly diminishing the pressure, but is not much affected by a gradual diminution. After gradual diminution of pressure a filter is obtained which does not markedly change in permeability on suddenly varying the pressure. The high concentration of the residual fluid is probably due to the water penetrating the gelatine easily, whereas the ferric hydrate is largely left on the surface of the gelatine, where it forms a concentrated solution of higher specific gravity than the rest of the fluid and so gives rise to convection currents which cause it partly to mix with the remainder of the fluid in the filter case. The candle retains a skin of colloidal ferric hydrate, and the filter most probably at the beginning of the filtration acted as a simple gelatine filter, but subsequently as a compound ferric hydrate gelatine filter.

### *Filtration of a Staining Colloid, Neutral Red, through* 11 *per cent. Gelatine under Varying Pressure.*

Preliminary experiments with horse serum and soluble starch showed qualitatively similar effects to those obtained with colloidal ferric hydrate, and it seemed probable that the majority of colloidal solutions would behave in a similar way on filtration through gelatine.

It seemed probable, however, that those colloidal solutions which stain gelatine would show considerable difference in behaviour.

*Method.*—A 0·5-per-cent. solution of neutral red in distilled water was pressed through 11 per cent. dialysed gelatine and the content of the fractions of filtrate, etc., determined colorimetrically.

*Examination for Neutral Red.*—Table II.

Original Fluid: 0·5 per cent. neutral red = (100) N.R.

Filtrates: The 1st, 2nd and 3rd fractions showed (0·0), (2·3) and (0·04) respectively. A sudden diminution in pressure from 100 to 20 atmospheres caused a slight decrease in the value of the filtrate (0·03). On raising the pressure once more to 100 atmospheres, the succeeding fractions gradually increased in content up to (4·68) N.R.

Residual Fluid: The colour intensity was much diminished and indicated only (18·75) N.R.

*Conclusions.*—The gelatine filter is slightly permeable at the beginning of filtration to neutral red, but the permeability decreases, as in the case of colloidal ferric hydrate, on continued filtration at constant pressure. Diminution of pressure has the opposite effect to that obtained with sodium chloride and ferric hydrate as the permeability tends to decrease. The filter gradually becomes stained throughout and the permeability increases correspondingly.

### *Filtration of a Staining Crystalloid, Iodine in Potassium Iodide, through 11 per cent. Gelatine, under Varying Pressure.*

Iodine was chosen as a crystalloidal substance having the property of staining gelatine, and potassium iodide was used as its solvent in water. The interest of the experiment centred chiefly in the relative behaviour of the two substances and the mutual influence they exert on each other during filtration under varying pressure.

*Method.*—A solution of 0·214 per cent. iodine in 1·54 per cent. potassium iodide was filtered through 11 per cent. gelatine in 1·54 per cent. potassium iodide. The content of the fractions of filtrate, etc., was determined by titration with sodium thiosulphate, using starch as an indicator for the iodine and with silver nitrate, using potassium chromate as indicator for the potassium iodide.

*Examination for Iodine and Potassium Iodide.*—Table II.

Original Fluid: 0·214 per cent. $I_2$ = (100) $I_2$. 1·54 per cent. KI = (100) KI.

Filtrates: The 1st c.c. contained a considerable percentage of iodine (5) and likewise of potassium iodide (68·8). On continued filtration at constant pressure the iodine concentration rose steadily to (25); the iodide rose slightly and then fell to (68·3). On suddenly diminishing the pressure from 100 to 15 atmospheres the iodine concentration diminished and the iodide increased. On raising to 100 atmospheres the iodine rose, whereas the iodide rose and then fell in concentration. Removing the pressure for 12 hours the few drops of liquid which percolated through contained no iodine and only (43) of iodide. Re-establishing the pressure of 100 atmospheres the iodine content rapidly increased, whereas the iodide in the first few drops was abnormally high (93·5), but decreased in the second fraction of 6 c.c. to (86·0). Once more removing the pressure for three hours the iodine diminished to (1·0) and the iodide to (34·4), confirming the former result. The pressure was now slightly raised, viz., to 10 atmospheres, when the iodine became (25) and the iodide reached the highest value so far (94·6). A further rise in pressure to 100 atmospheres increased the iodine to (30) and diminished the iodide to (92·5). Variation between 100 and 50 atmospheres did not further affect the iodine content but did influence the iodide. Finally, a gradual fall in pressure to 25 atmospheres caused both iodine and iodide to diminish in concentration.

Residual Fluid: The concentration of iodine was only (30), whereas the iodide was (109·7).

*Conclusions.*—The gelatine absorbs a large amount of iodine, but is easily permeable to the same and the permeability rises and falls, as in the case of neutral red, with rising and falling pressure. The increasing concentration of iodine in the gelatine increases the power of the latter in retaining potassium iodide. On entirely removing the applied pressure the gelatine robs the liquid percolating through almost entirely of its iodine and the iodised gelatine abstracts about two-thirds of the iodide.

The filtrates, after a drop in the pressure, show an increased amount of iodide on recompression similar to the effect observed in the filtration of sodium chloride.

### *On the Influence of the Nature of the Solution and of Varying Pressure on the Rate of Filtration.*

A few preliminary measurements of the rates of filtration of various solutions were made to elucidate some of the physical properties governing the action of the gelatine during filtration.

*Method.*—As a rough approximation the number of drops per minute falling from the nozzle of the filter case was taken as a measure of the rate of filtration. The rate was determined after the first 2 c.c. had passed, and again after about 50 c.c. in all had been collected.

*Rates of Filtration.*—Table III shows that distilled water, 0·8 per cent. sodium chloride and megatherium lysin pass 9 per cent. gelatine in the initial stage more rapidly than in the final under constant pressure. Solutions containing 0·214 per cent. iodine, with 1·54 per cent. potassium iodide, and 5 per cent. colloidal ferric hydrate pass 11 per cent. gelatine with similar variations in rate. For all these solutions the decrement in rate is practically the same. On filtering horse serum in full strength, however, the decrement in rate was found to be abnormally large.

Table III.

| 100 atmos. 12° C. | NaCl. | KI, I. | NaCl lysin. | NaCl toluol. | NaCl Am.Alc. | NaCl HBū. | Dist. water. | Coll. $Fe(HO)_3$. | Serum. |
|---|---|---|---|---|---|---|---|---|---|
| Initial drops per minute | 7 | 7 | 8 | 16 | 18 | 6 | 9 | 6 | 6 |
| Final drops per minute | 4 | 4 | 5 | 18 | 60 | 18 | 4 | 4 | 1 |

A solution of 0·8 per cent. saline containing toluol as a fine emulsion did not show any decrement.

On the other hand, 0·8 per cent. saline containing either amylic alcohol or butyric acid (4·8 per cent.) gave very distinct increments in the rate of filtration.

Table IV gives a comparison of the rates obtained for distilled water and 0·81 per cent. sodium chloride containing 4·8 per cent. of butyric acid. It is evident that distilled water filters more and more slowly, whereas the saline butyric acid passes more and more rapidly. The effect of removing the pressure for two minutes enables the distilled water on re-establishing the pressure to pass through at a higher rate, and if the pressure be removed for 10 minutes the gelatine regains completely its original rate of filtration.

Table IV.

| Distilled water. | | | | Sodium chloride and butyric acid. | | | |
|---|---|---|---|---|---|---|---|
| Pressure in atmos. | Time in minutes. | Drops per minute. | C.c. per minute. | Pressure in atmos. | Time in minutes. | Drops per minute. | C.c. per minute. |
| 100 | 0 | 7 | — | 100 | 0 | 6 | — |
| 100 | 13 | 6 | 0·5 | 100 | 2 | 5 | — |
| 100 | 21 | 5 | 0·5 | 100 | 5 | 9·5 | — |
| 0 | 23 | 0 | — | 100 | 7 | 11 | 0·7 |
| 100 | 29 | 6·5 | 0·6 | 95 | 15 | 9·75 | 0·6 |
| 100 | 32 | 5 | 0·5 | 90 | 20 | 9 | — |
| 100 | 40 | 4·5 | 0·3 | 100 | 23 | 11 | 0·6 |
| 0 | 50 | 0 | — | 100 | 35 | 13 | 0·6 |
| 100 | 73 | 9 | 0·7 | 100 | 42 | 14 | 0·7 |
| 100 | 83 | 5·5 | 0·5 | 100 | 49 | 15 | 0·7 |
| 100 | 133 | 4·5 | 0·4 | 100 | 64 | 16 | 0·8 |
| | | | | 100 | 71 | 18 | 0·9 |
| | | | | 100 | 74 | 18 | 1·1 |

*Conclusion.*—Solutions of salts such as sodium chloride, or colloids such as ferric hydrate, will not appreciably modify the rate of filtration through gelatine unless in concentrated solution. Amylic alcohol and butyric acid accelerate the rate of filtration.

It seemed possible that this effect might be due to the condensation of amylic alcohol and butyric acid on the walls of the gelatine pores, and that the change in viscosity might account for the increased rate. This, however, does not seem probable, for a toluene emulsion did not markedly affect the rate of filtration, and as the viscosities of the toluene and amylic alcohol used were found to be 0·49 and 4·4 respectively when compared with distilled water as unity, one might conclude that the toluene would accelerate, and the amylic alcohol retard, the rate of filtration.

On the other hand, the action of amylic alcohol and butyric acid is consonant with the view that the surface forces between gelatine and saline are modified. The surface tension of saline against air diminishes with increasing concentration of both amylic alcohol and butyric acid. Both of these substances markedly influence the rates of sedimentation of solid suspensions through water,* and this fact seems to have a close connection with their influence on the rate at which water passes through a porous solid or jelly such as gelatine.

The effect of continued pressure on gelatine seems to be a tightening of the pores, which is nearly complete at 100 atmospheres in 40 minutes, and decompression apparently allows the pores to resume their original dimensions in about 10 minutes at atmospheric pressure.

### *On the Expression of Water and Gelatine from a Wet Filter.*

In agreement with E. W. Reid, the filtrates were found to contain gelatine, but in variable amounts. Thus the first fraction of about 4 c.c. usually contained most, and after 20 c.c. had passed the gelatine was only present in traces. Rough estimations with tannic acid indicated about 0·5 per cent. gelatine in the first, and less than 0·1 per cent. in the fifth fraction on filtering distilled water. These contents of gelatine were not found to influence the titrations given above.

It has been hitherto assumed that the water in the pores of a wet filter is largely expressed before the liquid undergoing filtration appears in the filtrate undiluted by the same. For this reason Martin† discarded the first 10 c.c. of filtrate, and E. W. Reid‡ concluded that the concentration of even a second fraction of 25 c.c. might be considerably reduced by this dilution. It seems to me, however, that the effect of the water in the gelatine on the concentration of the filtrate is neither so marked nor so long continued as has been imagined, for the following reasons. In the first place, the filtrates from saline through gelatine containing the same concentration of saline are at first only about half as concentrated as the original saline. Secondly, on decompression, and again filtering, the filtrate may contain a higher concentration of salt than the original saline. This seems conclusively to show that much of the dilution of the filtrate is due not to the water of the filter, but to the retention of the salt by the gelatine. Further, Reid found the quantity of water in freshly prepared filters to vary between 2 and 13 grammes—*i.e.*, presumably, the amount which can be removed by dry air. It is probable that only a small part of this water will be removed by the passage of a slow current of liquid through the gelatine, the remainder being retained by adsorption or inbibition forces, etc. In this connection an observation which was made with all the filtrations given in this paper may not be unimportant. It was found that the sum of the volumes of the filtrate fractions and residual fluid in any one filtration experiment was less than the volume of the original fluid introduced into the wetted apparatus. It is a well-known fact that water can be forced into gelatine by pressure, and as a large part of the gelatine in the pores of the filter, during filtration at 100 atmospheres, must be under considerable pressure, it seems reasonable to assume that part of the original water of the wet filter is more firmly bound, and that about 5 c.c. are imbibed partly from the water of the filter and partly from the fluid filtered. The rapid passage of iodine, neutral red, and colloidal ferric hydrate into the filtrate also point to a considerable percentage of the first fractions of filtrate being contributed by the fluid filtered. It seems, on the whole, as if the free water of the filter is almost wholly removed in the first fraction of 5 c.c. filtrate.

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* *Cf.* Craw (1904).

† *Loc. cit.*

‡ *Loc. cit.*

### *The Application of Mechanical and Adsorption Hypotheses to the Filtration Phenomena of Gelatine.*

*Mechanical Hypotheses.*—The most obvious explanation of the retention of colloidal substances by the gelatine filter is that the colloidal molecule or grain is too large to pass through the pores. Martin* has advanced the view that the non-filtration may be due either to the size of the molecules or to some interaction between the colloidal membranes and the dissolved colloidal molecules. It has, however, been shown by E. W. Reid (1904), Gatin-Grazewska (1904), and others that proteids, glycogen, and other typically colloidal substances, exert no measurable osmotic pressure in solution, do not influence the freezing point of the aqueous medium, and in general diffuse very slowly, if at all. These facts lend much probability to Oswald's view that colloidal solutions may be regarded as very fine suspensions. As the molecular weights of these substances are as yet quite indefinite, it would seem more justifiable to speak of the colloidal "grain" than of the colloidal "molecule." Accepting this view of the nature of colloidal solutions, the action of the filter admits of an apparently satisfactory explanation on a purely mechanical basis.* During the filtration, for example, of colloidal ferric hydrate, Table II, the first fraction contained none of the colloid, and probably consisted chiefly of the water of the filter. In the succeeding fractions a little ferric hydrate passed through the larger pores, but these were speedily blocked by the particles. Under the pressure of 100 atmospheres the pores are probably compressed, and the effective passages are soon completely blocked by ferric hydrate. On suddenly diminishing the pressure the gelatine begins to return to its original condition, and the effective passages increase in number and diameter. The condensed ferric hydrate, no longer completely blocking the widened passages, is swept out of the gelatine by the oncoming fluid, and so gives a filtrate with an abnormally high concentration.

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* *Loc. cit.*

On the other hand, by gradually diminishing the pressure the ferric hydrate has time to fill the new and the gradually-widening pores, so that the filtrate contains only a trace of colloid. If the ferric hydrate be not expressed on recompression, it is conceivable that the compound filter of ferric hydrate and gelatine would be less affected as regards permeability by variation of pressure than the original gelatine. Much as this view has to recommend it, it does not seem to be the whole explanation, as it does not satisfactorily account for the phenomena observed with sodium chloride. It is highly improbable that sodium chloride is retained by a mechanical blocking of the passages, and that it is swept in high concentration into the filtrate, on decompression, because the condensed sodium chloride is no longer of the same dimensions as the widened passages. Recourse must, therefore, be had to some other view, which may considerably modify the explanation given for colloidal substances.

*Adsorption Hypotheses.*—Porous substances, powders, and fine suspensions of solids in aqueous media have the power of removing salts and other substances from solution. The action is attributed to forces of the same nature as those which give rise to adhesion and the wetting of a solid by a liquid, *e.g.*, of clean glass by water. The phenomenon usually called adsorption is common to all substances with highly developed surfaces, and the intensity of the adsorption depends not only on the physical condition but also on the chemical nature of the active surface. Gelatine being, probably, a porous substance, presents a large surface to the enclosed fluid, and it seems highly probable that when this fluid contains crystalloids or colloids adsorption will take place. Thus, gelatine containing 0·8 per cent. saline apparently retains about one-half of its content in sodium chloride, and the retention of crystalloids and colloids on filtration through gelatine at constant pressure seems to admit of explanation on the basis of adsorption. The results obtained in the investigation of the adsorption phenomena of the sedimentation of silts through aqueous solutions may therefore be applied to what apparently is the converse of that process, viz., the passage of aqueous solutions through porous solids or powders, under pressure. This is rendered highly probable from the fact that the rate of sedimentation of a silt through saline is generally accelerated by the presence of butyric acid, amylic alcohol, and other substances influencing surface tension, and similarly the rate of filtration through the porous gelatine is accelerated by the same substances. An additional factor may, however, come into play in gelatine filtration. The effect of pressure on gelatine containing a solution of salt will be to cause the absorption of water. It seems permissible to assume, in view of Hardy's work on the structure of gelatine, that this additional water will be taken up by the web mass and will probably lead to a diminution in dimensions of the pores or web spaces. This view would account for the continued diminution in rate of filtration at constant pressure. The process should show some similarity to the passage of a solution into filter paper, where the water passes in more readily than the substance in solution.

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* *Cf.* also M. Traube (1866, 1867). Traube regards precipitation membranes as atomic and molecular sieves.

A sudden diminution in pressure will lead to the rapid expulsion of the imbibed fluid, which will sweep the adsorbed matter into the widening pores. The gelatine itself will thus express part of this concentrated fluid, and at low pressures highly concentrated filtrates will be obtained. This will take place markedly when the adsorption is easily reversible, but the less reversible adsorptions of certain staining substances, *e.g.*, neutral red and iodine will not give this effect. Further compression and decompression would presumably lead to a more uniform distribution of the adsorbed substance throughout the gelatine, and as the filter is now also more saturated, the effect of variation of pressure on the concentration of the filtrate would be less marked.

### *Summary of Conclusions.*

1. Wet gelatine filters are to be preferred to those which have been partially dried, as the former have more uniform rates of filtration, and dilution of the filtrate by the water of the gelatine can be largely eliminated.

2. Under constant pressure the gelatine of the filter absorbs water, and its porosity gradually decreases; on decompression this water is expressed, and the original porosity is rapidly regained.

3. Gelatine of a definite concentration apparently has a specific permeability for different crystalloids and colloids; the value is high but not complete for the crystalloids sodium chloride, potassium iodide, and butyric acid, and it is low, but not zero, for the colloids ferric hydrate, serum and soluble starch.

4. As filtration proceeds the crystalloids pass through in increasing concentration, whereas the colloids rapidly decrease to zero.

5. The simultaneous filtration of two substances may influence their specific permeabilities, thus butyric acid alters the permeability to sodium chloride, and iodine that of potassium iodide.

6. Variations in the gelatine influence the permeability, *e.g.*, formalised gelatine is less permeable to sodium chloride than ordinary gelatine, and 15 per cent. gelatine is less permeable to megatherium lysin than 7·5 per cent.

7. Variation in the pressure causes remarkable changes in permeability. A sudden diminution of pressure gives highly concentrated filtrates of both crystalloids and colloids, whereas a gradual diminution has practically no effect.

8. Substances which stain the gelatine, *e.g.*, neutral red and iodine, give filtrates with lower concentrations on diminishing the pressure.

9. The rate of filtration is accelerated by amylic alcohol and butyric acid, which accelerate the rate of sedimentation of silts in a similar way.

10. Part of the phenomena may be explicable on the mechanical view of a blocking of the gelatine pores, but chemical relations between the gelatine and substances filtered must be taken into consideration, and probably the most satisfactory view is that the action of gelatine on the solutions tested is essentially an adsorption phenomenon.

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# *A Case of Regeneration in Polychæte Worms.*

By Arnold T. Watson, F.L.S.

(Communicated by C. S. Sherrington, F.R.S. Received October 23, 1905,—Read January 18, 1906.)

The facts recorded in the following note were ascertained in the course of my observations made upon a marine rock-boring polychæte worm found at Tenby in the spring of the present year. This worm, a species of Potamilla, is living in limestone rock, in which another species of the same genus, *Potamilla reniformis*, is also burrowing. It differs from the latter in various particulars, amongst which may be noted the absence of eyes on the branchial filaments, the colour of the blood (which is red instead of being sometimes green), the form of the setæ, and the character of the external tube, which is largely covered, especially at the tip, with minute pieces of shell attached edgewise, imparting to it a white, rugged appearance, somewhat similar to that of the tube of *Owenia*. The worm is sometimes as much as 3½ inches long, and is seldom extracted entire from the rock, fragments only, of varying length, usually being obtained.

It occurred to me that this material might be utilised for the study of the regeneration of the lost parts, and my experiments in that direction succeeded beyond my expectations. Not only did the fragments renew these parts (both anterior and posterior) but they demonstrated the existence of a power to economise labour in this respect, by changing the arrangement of certain of the *old parts*, so as to complete the model of the original animal.

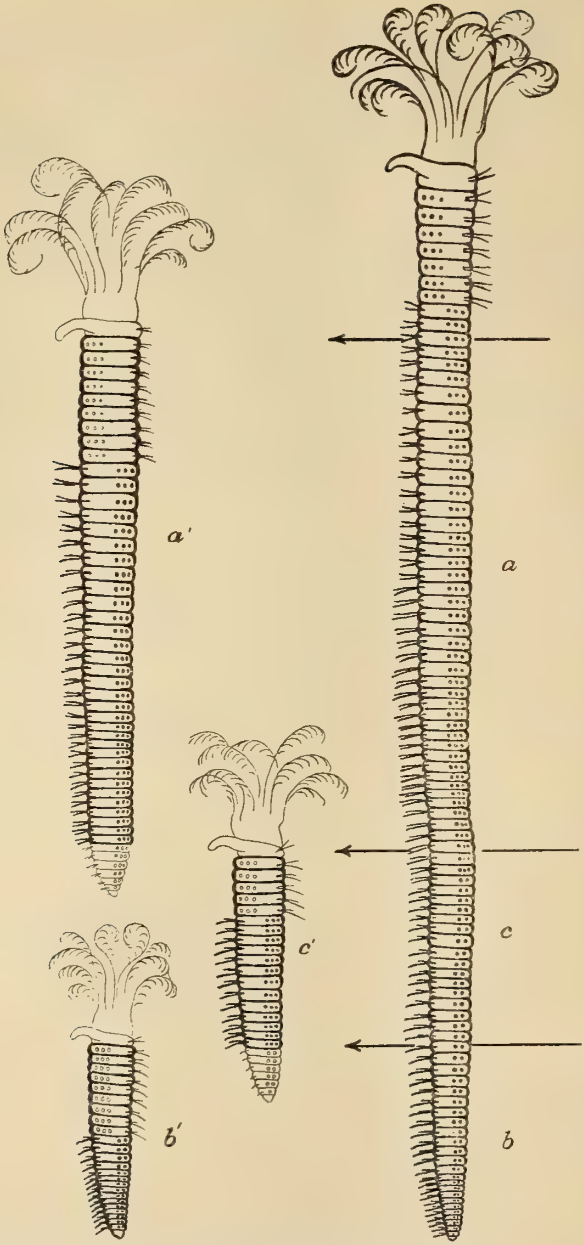
The body of the worm in question consists of a large number of segments, all of which, with the exception of those at the two extremities, are endowed with a set of hooks (uncini) and bristles (setæ) on either side, and it is one of the characteristics of the Sabellidæ, the family to which this worm belongs, that the character and arrangement of these appendages in the anterior or thoracic portion differs from that in the posterior or abdominal part. The setæ in the former are situated dorsally and the uncini ventrally, whilst in the abdominal portion the uncini are dorsal and the setæ ventral. This arrangement, besides enabling the worm to rotate on its long axis in either direction at will, also facilitates the bringing of its thoracic ventral glandular plates and collar-lobes into contact with the inner surface and top of the tube, and is probably connected with the tube-forming habits of the worm. Some of the fragments which I have had under observation were without head and thorax, and consisted of abdominal segments only, the setæ and uncini consequently being ventral and dorsal respectively, from end to end.

In cases of regeneration my experience has been that new segments are much more freely produced at the posterior than at the anterior extremity of the body, and the problem which occurred to me was, *how*, and in what length of time, the thoracic segments (about eight in number), with the inverted arrangement of chætal appendages, which is needful to the life-work of the worm, would be reproduced. The answer came in the nature of a surprise. Beside the cephalic plume-bearing segment, *one new* setigerous *thoracic segment only* was formed, but the chætal plan of the succeeding five or nine abdominal segments was changed; the dorsal uncini in these segments first disappearing gave place to setæ, and later the ventral setæ were replaced by uncini; the new setæ and uncini, moreover, were changed to the forms characteristic of this part of the body. In other words, so far as the chætal plan is concerned, a new thorax had been constructed from the abdominal segments. How far the internal structure has been affected by the change remains to be ascertained.

The observations extended over a period of five weeks, and were made upon two portions of apparently one and the same worm. One portion (comprising the two parts marked *a* and *c* on the figure), about $\frac{3}{4}$ inch long and consisting of 69 abdominal segments, being minus head and thorax, as well as the anal, and numerous preanal segments; the other part, *b*, $\frac{1}{8}$ inch long (probably the hinder portion of *c*), consisting of 36 preanal and the anal segment.

In order to expose a large surface of water to the air and bring the animals as near thereto as possible, the experiments were carried on in watch-glasses, fresh sea-water being supplied twice daily. The first fragment, *ac*, in course of a day or two, attached itself to the watch-glass by means of a narrow cord or loop which it secreted, and which served, by a constant twisting movement of the animal, to sever the portion, *c*, $\frac{1}{8}$ inch long, and consisting of 18 of the posterior segments. The number of parts available for observation was thus increased to three. Each was placed in a separate watch-glass, to which it attached itself slightly by a secretion from the ventral surface, and each part succeeded in changing the chætal arrangement of certain abdominal segments into that of thoracic ones, as follows—in *a*, nine segments became thoracic; in *c*, five; and in *b*, nine. The normal number of thoracic segments in the few adult specimens which have passed through my hands is eight (in one case it is nine), but the number appears to vary considerably in any given species of Sabellid.

The number of abdominal segments transformed is possibly, to some extent, regulated by the total number of segments contained in the fragment undergoing repair, but it may also be dependent upon the number of thoracic



Diagrammatic Outline of entire Worm, viewed from left side, showing dorsal setæ and ventral uncini (indicated by dots) in the anterior or thoracic portion, and the inverted arrangement (dorsal uncini and ventral setæ) in the much longer abdominal part. The portions referred to are indicated by the corresponding letters, *a*, *b*, *c*, in the diagram. Points of fracture shown by arrows.

Figures to left, *a'*, *b'*, *c'*, show respective fragments as restored. New growths (head and posterior) and setæ and uncini of converted (abdominal into thoracic) segments shown by thin lines and small circles.

segments possessed by the worm from which the fragment was derived. I am, unfortunately, unable to make any comparison in the latter respect as regards the examples referred to above, the anterior portion of the worm not having been secured. The constancy of the figures is, however, very striking, and it is possible that the higher numbers might correspond with that of the thoracic segments of the worm from which the fragments came. This suggestion is supported by what is now taking place in a similar experiment which I am making on another Sabellid (*Sabella pavonina*). In this case the number of abdominal segments transformed corresponds *exactly* with that of the thoracic region of the worm from which the abdominal segments were excised.

It seems highly probable that the power of adaptation, described in this paper, is possessed in greater or less degree by all the abdominal segments and from experiments which I have now in progress and from the observations made by MM. C. Vaney et A. Conte on *Spirographis spallanzanii*,* it is evidently possessed, also, by other members of this family of worms. The results obtained by MM. Vaney et Conte agree very closely with my own. In their experiments the worm was extracted from its tube and a ligature was placed round the body at any desired point. The worm was then restored to its tube and exposed in the sea. In due course, fission at the point ot ligature was effected, followed by renewal of the anterior and posterior parts and transformation of a number of the abdominal segments to form a new thoracic region.

In MM. Vaney and A. Conte's paper attention is called to the fact that worms are frequently captured which have evidently been produced by the regeneration of fragments, whilst other specimens (a living one is now in my possession) show marked constrictions in certain points as though produced by ligature. They suggest that these constrictions are probably followed by fission and regeneration, and that the worm is able, naturally, to produce scission, but by what means they are unable to say.

The observation recorded in this paper, which is supported by a similar one in the case of the *Sabella pavonina*, above mentioned, appears to supply the needed solution. It has thereby been shown that the worms can themselves provide ligatures and so sever the body at any given point. This power is doubtless of importance in the preservation of the individual, since diseased conditions of the tubicolous worms are usually more common at the anterior than the posterior extremity, consequently an excision and ablation of the anterior part preserves the existence of the worm.

The species which I have had under observation, like several others of

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* 'Compt. Rend. d. la Société d. Biologie,' Paris, 1899, p. 973.

this group, appears, under artificial conditions, unable when expelled from its burrow to re-establish itself in a new home. It is, therefore, interesting to enquire what influence evokes the reconstruction of the abdominal segments, alike whether the fragment be confined within its tube or free as in a watch-glass.

# *On the Cytology of Malignant Growths.*

By J. Bretland Farmer, F.R.S., J. E. S. Moore, A.R.C.S., and C. E. Walker.

(Received November 17,—Read December 7, 1905.)

[Plates 8—12.]

In the winter of 1903 we presented to the Royal Society a preliminary account of the results of our investigations on the cytology of malignant or cancerous growths, in which we stated that we had recognised a certain type of nuclear division, known as the heterotype, to occur in the cells of these pathological tissues. Hitherto, this peculiar kind of mitosis, characterised *by a reduction to one-half of the normal number of the somatic chromosomes,* together with highly characteristic forms of their bodies themselves, had only been known to occur in connection with the so-called reduction division, that, in animals, immediately precedes the formation of the sexual elements. These reduction divisions constitute a well-known phase in the life cycle of all higher animals and plants, consisting invariably of two consecutive stages, which are distinguished as heterotype and homotype mitoses respectively. As these two mitoses constitute so well-defined a stage in the cellular life history of the higher organisms, we have proposed to emphasise this fact by the introduction of the term "maiosis," designating the stage itself as the maiotic phase. Thus the heterotype and homotype mitosis form respectively the first and second maiotic division.

We propose, in the present paper, to deal more fully with the cytological details of malignant growths, in so far as we have been able to investigate them, and we shall endeavour briefly to point out the conclusions that we think may legitimately be drawn from them. In doing this we desire, as far as possible, to confine ourselves to a consideration of the evidence we have been able personally to obtain, and we propose to avoid a general discussion of the numerous theories as to the ætiology of the disease, and especially of those that are based on clinical experience, except in so far as our own observations seem to throw light on those matters. We are fully aware that in pursuing such a course, we lay ourselves open to the charge of incompleteness, and of unduly neglecting the views of others. But an attempt to deal at all adequately with the vast literature that has grown up around the subject of cancer would involve a very lengthy, and probably not a correspondingly fruitful, discussion.

We further propose to confine ourselves to a consideration of carcinomata, and we do not intend to deal with sarcomata at this juncture, since we have enjoyed far fewer opportunities of examining growths of this class with anything approaching to completeness. On the other hand, we have been fortunate in securing a large number of carcinomata of very different ages, for which we are indebted to the kindness of several London Surgeons and others. We also desire especially to acknowledge our indebtedness to the Committee of the Imperial Cancer Research Fund for a grant in aid of our investigations.

We have been able to study extremely early stages in cases of cancers of the rectum, scrotum, penis, lip and tongue. A similarity in all essential features was plainly apparent in the history of the cellular evolution of each of these growths respectively. It will, therefore, be sufficient for the purpose of this paper, and it will also conduce to brevity, if we continue our account, especially to one of them, merely premising that it represents a type to which the rest really conform in all essential details of interest in the present connection. We will, therefore, select as our chief example a very early case of rectal epithelioma. This was not only a very young, but also a very actively proliferating growth, and it was preserved immediately after excision (see Plate 8, fig. 1. This is also to be compared with Plate 9, fig. 2, which illustrates a young growth of epithelioma of the penis).

For the sake of clearness it will be useful to give a short preliminary description of the mucous and subjacent layers of a healthy rectum as they appear under high magnification. The boundary of the lumen of the gut is composed of a large celled columnar epithelium, the elements of which are generally set one row deep upon the basement membrane. The latter is formed from the connective tissue and other elements of the submucous layer. All over the interior surface of the intestine the mucous epithelium is produced outwards into folds that bound correspondingly finger-like cavities, the latter forming the so-called crypts of Lieberkühn. As seen during ordinary states of activity, the epithelium is composed of elongated cells, each consisting of a mass of granular protoplasm in which a large nucleus is situated. The position of the latter in the cell is subject to individual variations. Amongst these resting cells are others in which the cytoplasm contains masses of secretion, and these constitute elements commonly known as goblet cells. In between the cells of such a rectal epithelium are always to be seen a few wandering elements which are apparently migrating from the submucous into the mucous layer and *vice versâ*. Their number is, however, very limited, and they either resemble the white corpuscles of the blood, or their nuclei exhibit a lobed appearance which seems to represent a stage of fragmentation.

In the processes of the submucous layer which interdigitate with the crypts, we find, in the first place, a kind of framework or loose scaffolding of intestinal connective tissue, between the strands and sheets of which can be seen the loops of the anastomosing rectal arteries and veins. Within and without this region, but especially in the centre of the projections of the submucosa, vast numbers of lymph bodies are always conspicuous, the central mass or core of the latter marking the termination of the irregular lymph sinuses and vessels (fig. 1, *h*).

Amongst the columnar cells of the mucous layer during states of ordinary activity we occasionally encounter nuclei in various stages of ordinary mitosis. More rarely nuclei in process of fragmentation or amitosis may be seen. Neither kind of division is frequent, and the tissue seems to be merely regenerating itself by the replacement of individual cells as fast as these disappear. Amongst the leucocytes and lymph bodies, as well as occasionally amongst the connective tissue corpuscles, division is also plainly to be discerned. The nuclei of the connective tissue elements divide mitotically, whilst in the leucocytes we meet both with true mitosis and with those peculiar forms of leucocytic fragmentation that have already been described and figured by other writers, and are generally well recognised. Wherever we light upon true mitotic figures, whether in the mucous cells, in the connective tissue elements, or in the lymph bodies, the phases of division invariably agree with the type of mitosis characteristic of the non-reproductive portions of the body. They are typically somatic or premaiotic.* In every mitosis of this nature the chromosomes emerge from the resting nucleus in the form of elongated or bent rods, and in the ordinary premaiotic number (32).

During the stage of the equatorial plate each of them is easily seen to be longitudinally split, the two halves passing respectively to the opposite poles of the spindle to contribute to the formation of the two daughter nuclei. Under ordinary conditions the above cytological conditions and appearances remain unchanged in the rectum.

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* See Farmer and Moore, "On the Maiotic Phase (Reduction Divisions) in Animals and Plants," 'Quart. Journ. Micr. Sci.,' vol. 48.

Having completed that foregoing brief survey of the structures and changes that occur in the cells of a healthy rectum, we are in a position to consider the features that arise on the early development of a cancer in this region. In the case of the neoplasm we are especially concerned with, the area involved was very small, barely a centimetre in diameter, and this area marks the original seat of the disease. The central portions were but slightly ulcerated or broken down, whilst the margins were hardly at all raised. The essential details of the structure as shown near the edge of the growth are figured in fig. 1.

Towards the periphery of the growth the columnar cells are scarcely displaced, but they exhibit a more or less altered appearance when compared with the still healthy cells in their vicinity. There can, in fact, be traced a narrow and not very sharp line of demarcation that distinguishes the cancerous from the non-cancerous epithelial elements. A consideration of the structure of the cells in this region makes it perfectly clear that the growth has not proceeded from a more remote centre to invade the healthy mucosa, but that the cells of this layer are themselves assuming the peculiar characters of the growth. In other words we are confronted with a primary transmutation of normal and functional cells into those of cancerous tissue. The tumour was small and flat, the change visible at its margin having presumably proceeded centrifugally over the more developed central area.

Thus the growth, regarded as a whole, must be considered as having originated from a relatively large number of functional epithelial cells by a direct conversion of them into neoplastic elements. No other interpretation seems reconcilable with the facts of the case, but we may defer the theoretical conclusion involved therein for subsequent consideration.

But, notwithstanding the evidence for the marginal spread of the growth by a direct alteration of the cells in this region, when once the change has been effected in them, the cancerous cells begin on their own account to invade the deeper layers of tissue situated beneath the epithelium. This is illustrated by fig. 1, *g*. The general nature of the process of invasion is so well known as to call for no specially detailed description here. It will be noted, however, as shown in the illustration, that the ingrowing cancerous tissue long retains many of the features characteristic of the particular epithelium from which it has sprung. This is, of course, not uncommon, especially in the case of glandular tumours (fig. 1, *b*).

The marginal zone of demarcation between the diseased and healthy tissue is distinguished at its periphery by a barely perceptible increase in the size of the elements that compose it. Immediately within this outermost limit a rapid multiplication of the cells is seen to be taking place, and even in the second or third cell from inside the margin the altered character is easily recognised (fig. 1, *b*). The cells exhibit an increase in cytoplasm, a comparative absence of secretory activity, and a peculiar and well-defined change in the appearance of the nucleolus. The alteration in this last-named structure consists in its larger size and denser appearance. Furthermore, very many nuclei are to be seen in a state of active division. Whilst some exhibit various stages of mitosis, others are clearly undergoing fragmentation or amitosis.

At this stage of the development of the tumour, the peripheral cells that are dividing mitotically show all the characters of ordinary premaiotic divisions, and the normal number (32) of chromosomes can frequently be counted with certainty (figs. 5 and 10). But concomitantly with the first changes indicated in the epithelial cells at the edge of the neoplasm, a marked activity may be observed to take place on the part of the leucocytes. These bodies are seen to be in a condition of active migration and multiplication, much like that which occurs during the early stages of simple inflammation. In the subsequent stages, however, the early parallelism with inflammatory processes is lost, and there supervenes a remarkable phase in the further development of the cancerous cells. Not only do the cells of the tissue in question multiply with great rapidity, whilst the leucocytes amongst them are enormously increased in number, but the latter are seen not infrequently to force their way into the cancer cells, particularly in the so-called "giant cells," where, however, they are still to be recognised with ease and certainty (figs. 1, *x*, *d*, 11, 12, 13). This circumstance has already been noticed by others, but we have been led to attach a somewhat special importance to its occurrence. Some writers have suggested that the cancer cells are acting phagocytically upon the leucocytes, but, as a matter of fact, the further sequence of events indicates that the cancer cells are no more to be regarded as attacking the leucocytes than the latter as destroying the cancer cells. There can be no possible doubt that the leucocytes actively force their way into the elements in question. They may not seldom be observed to be in close juxtaposition with these, or in a hollowed depression, or finally they may be discovered just within the cell membrane, where they are easily recognised on account of their characteristic nuclei (fig. 11). They show no sign of disintegration—at least, in the great majority of cases—and the fact that they may persist for a considerable time without destroying the cell into which they have invaded, is proved by examples in which a leucocyte lying in the cancer cell is seen to be surrounded by several nuclei that have clearly originated by the fragmentation of the original cell nucleus, and, indeed, one of these is shown to be still dividing amitotically.

But the strongest proof of the persistence of the leucocyte under these remarkable conditions is afforded by the cases, not few in number, in which we have been able to trace the leucocyte actually dividing within the cancer cell (figs. 12, 13). Of course, it is only during the early stages that it is possible to be certain that a second dividing nucleus in a mass of protoplasm belongs to a leucocyte, and does not represent mitosis in a small nucleus that has arisen by fragmentation. But we have seen so many cases of early stages of leucocytic mitosis within the cancerous (or "precancerous") cell that it seems impossible to resist the inference that many of those frequently occurring cases in which a small nucleus is seen in the later phases of mitosis within the large nucleated cancer cell are to be attributed to this source. The nuclei of the cancer cell and leucocyte often divide simultaneously, and the two nuclear figures may also coalesce more or less intimately, and thus a commingling of leucocytic and epithelial chromosomes occurs on a spindle that becomes common to the two nuclei concerned. The cells so affected were, as already stated, usually the very large (giant) cells so characteristic at this stage of the development of the tumour, and we found that more than one leucocyte might enter and persist in a single cancer cell. In the earlier stages, of course, there is no difficulty in clearly recognising the intruding cell, since it retains its own cytoplasm and limiting membrane intact (see fig. 11), and the highly characteristic structure of the nucleus enables it to be identified even after these criteria have ceased to exist.

In the same region in which this series of events is proceeding a number of cancer cells are to be seen in various phases of mitosis, and, both in the aster and diaster of such nuclei, larger numbers of chromosomes were often encountered than are proper to normal somatic cells. These increased numbers are partly to be ascribed to the pluripolar mitosis distinguished by Hertwig and by Von Hansemann, and they result from the simultaneous mitosis of a number of nuclei lying in a common cytoplasmic mass.

But the observations recorded above indicate that, in the addition of leucocytic nuclei to those of the actual epithelial cells, we have confronted, at any rate, with one of the sources to which these excessive numbers of chromosomes (hyperchromatic nuclei of Von Hansemann) may be attributed, although a large number of the cells continue to multiply in the manner already described, it may also be seen that there exists a very considerable amount of amitosis, or direct nuclear divisions in the cells of the young parts of the tumour. There appears to be no evidence which would point to the conclusion that amitosis is in any way bound up with degeneration, or diminishing activity in those cells in which it occurs. Elements that have previously multiplied by amitosis and by fragmentation have given rise to the highly characteristic multinucleate cells, may again assume the mitotic method of increase, and *vice versâ*. A curious feature in the further division of these multinucleate cells, or syncytia as they may, perhaps, be more appropriately termed, is seen in the almost invariable circumstance that, on the resumption of mitotic activity, all the nuclei are in exactly the same phase.

This simultaneous character of the process is one which is shared by many other syncytia, *e.g.*, the myxomycetes. In these organisms, the nuclei are commonly observed not only to be dividing simultaneously over a considerable area of the plasmodium, but they also exhibit identical phases of the process at any given time. In examples of this simultaneous mitosis within the neoplastic syncytia, it often happens that the spindles of some, or even all of the dividing nuclei, become more or less intimately fused together, and in this way various forms of pluripolar mitosis are produced. Probably these pluripolar divisions owe their origin chiefly to the cause just indicated.

The figures produced are extremely variable, and it not unfrequently happens that, whilst the chromosomes belonging to the different nuclei are aggregated in the centre, the poles of three or more of the spindles involved are quite separate. In other examples the groups of chromosomes do not coalesce, but each equatorial plate is quite distinct, and lies in a plane different from that occupied by the equatorial plates of the other spindles. But when a more intimate fusion of the ends of two or more spindles takes place, it is obvious that the daughter nucleus formed in relation to such unions will receive an excessive number of chromosomes.

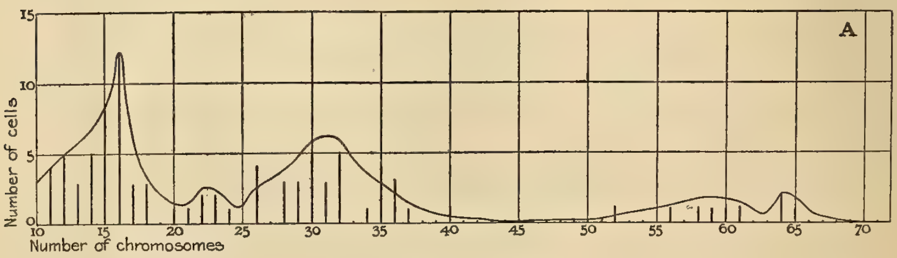
We would call special attention to the fact that giant cells of this character, also containing several nuclei, are present not only in the normal human testis, but also in the so-called red bone marrow, and that pluripolar mitosis may occur in such cells in a manner precisely similar to that so characteristic of cancerous tissue. The divisions of these early cancerous cells also exhibit other characters likewise encountered in the cells of the testis. Very often the daughter chromosomes do not move regularly towards the poles, but some either stray out of the direct line, or in other ways occupy unusual positions. These figures are also well known to occur in the heterotype division of some spore mother-cells of plants. In yet other examples of divisions in cancerous tissues, we have confirmed the observation of Von Hansemann that some of the chromosomes, as they are passing to the spindle poles, get ahead of their fellows, and form isolated or grouped chromatic particles that look as if they are about to be left out in the cytoplasm when the daughter nuclei become reconstituted. These figures are also paralleled by similar occurrences that may be seen in the cells of the testis, and they are known to occur during the maiotic divisions of some plants.

It is thus evident that hyperchromatic nuclei, that is, nuclei containing an excessive number of chromosomes, may be produced in at least two ways: firstly, by the inclusion of leucocytes, and the incorporation of the chromosomes belonging to these bodies with those of the cancer cells when mitosis sets in; secondly, through the formation, whether by amitosis or mitosis, of multinucleate syncytia, and by the subsequent confusion and mixing of the chromosomes originally belonging to two or more of the nuclei when the equatorial plate stage is reached.

These aberrant modes of division are found to proceed concurrently with the normal somatic mitoses that are going on in other cells in their immediate vicinity. It is impossible to say definitely whether there may exist any sort of alternation between the two types, though we are inclined to think that such is not the case. It is, however, important to notice that all the mitoses described above, whether they are normal in the number of chromosomes or not, agree in conforming to the somatic type of division. That is to say, no matter how many or how few the number of chromosomes involved may be, the spireme eventually divides into a number of rod-like elements, each of which splits longitudinally, and the daughter chromosomes resulting from such fusion are severally distributed between the daughter nuclei finally produced. In such typical cases this of course means that each of the two daughter nuclei receives one longitudinal moiety of such original chromosome.

But as we pass inwards from the growing edge of the tumour we encounter cells in which the nuclei exhibit important deviations from the ordinary somatic type of mitosis, and exhibit the characters otherwise met with during the heterotype division (*cf.* figs. 6, 7, 8). In the early stage of the phase of such nuclei the spireme exhibits that characteristic bunched appearance recalling the well-known contraction figure that is normally to be seen at the onset of the maiotic phase, that is in the prophase of the heterotype mitosis, in animals and plants. In addition to this, we have been able to ascertain that at about the same stage the spireme thread exhibits the longitudinal fission (fig. 6) that is highly characteristic, though perhaps not exclusively confined to the prophase of the heterotype division. The fission is especially well seen in those cases in which a marked polarisation of the spireme is apparent. But the most striking evidence of the validity of the comparison that we drew in 1903 between these particular nuclei and those of the reproductive cells during the maiotic phase of the animals and plants does not depend solely on the similar mode of evolution of the chromosomes from the resting nuclei in the "gametoid" cancerous and the true reproductive elements. The number of the chromosomes furnishes a far more important criterion. It is seen that a large number of dividing nuclei contains less than the normal complement of chromosomes. We have made a number of careful counts of the chromosomes in numerous cases of carcinoma, and always with the same result. In especial, we are indebted to Mr. L. Robinson for his assistance in this somewhat trying task. He has estimated the chromosomes in 400 dividing nuclei, taken (100 from each) near the actively growing regions of three different carcinomata originating respectively from the rectum, scrotum, penis, and in an example of deciduoma malignum.

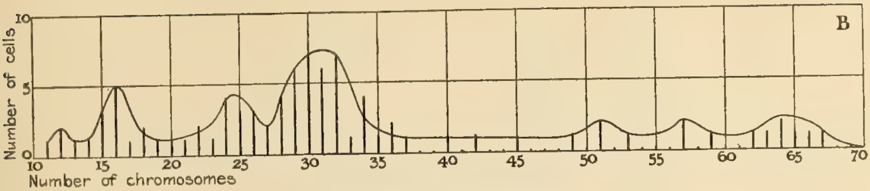
In every case we find two well-defined maxima, one set of nuclei containing 32, the other 16, chromosomes. For purposes of comparison he has counted chromosomes of the testis of the cockroach (Curve F), so as to obtain a control indicating the probable degree of accuracy represented by the estimations in the cancer nuclei. The same two maxima are, of course, apparent, but there is a similar average error around the maxima, due to the difficulty of the actual counting, and also the chance that some of the chromosomes might be absent from the section, or that a limited degree of variation may really occur. And, having regard to the fact that in the human species the chromosomes are not easy, even under favourable conditions, to estimate very accurately, whereas in the case of the cockroach the observer encounters far less difficulty in this respect, the results may, we think, be described as satisfactory. For although, after what we have said, it is obvious that, owing to amitosis, and especially to pluripolar mitosis, a considerable extent of variations is to be anticipated, the grouping of the numbers around the maxima of 32 (somatic) and 16 (reduced) is quite unmistakeable, as is shown in the accompanying curves.



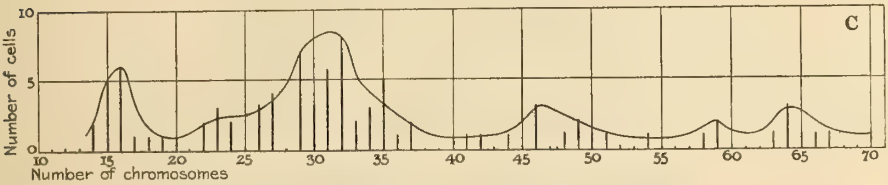
Carcinoma of Rectum.

The ordinates represent the number of cells that contained any given number of chromosomes, as indicated by the abscissæ.

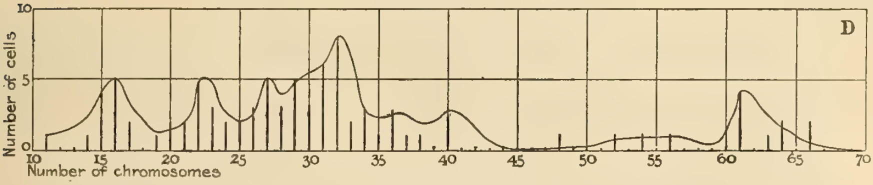
A. Cancer of the Rectum.—The hypochromatic nuclei to the left somewhat obscure, the maximum at 16. The grouping of numbers about 24 and 64 are fairly well shown.



Epithelioma of the Scrotum.



Epithelioma of the Penis.

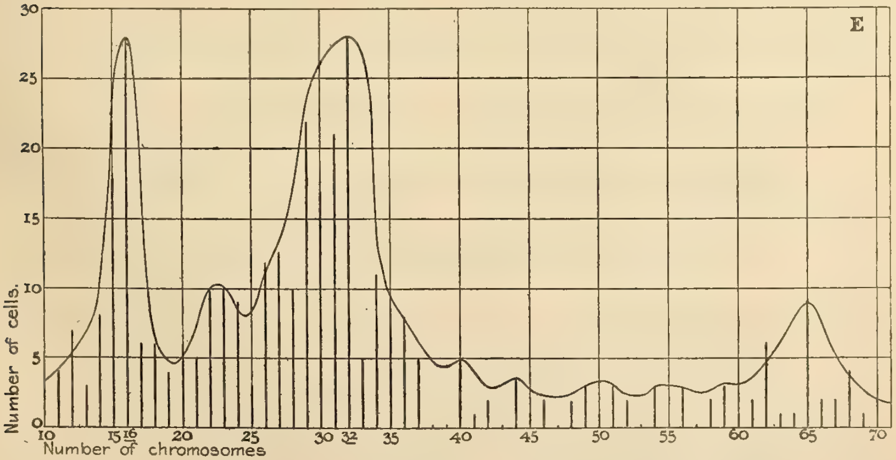


Deciduoma malignum.

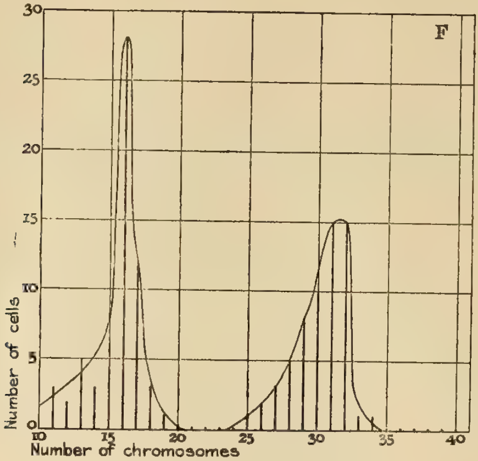
B. Epithelioma of the Scrotum. The maxima about 16, 24, and 32 are distinct, that about 64 not so clear.

C. Epithelioma of the Penis.—The maxima in the regions of 16, 24, 32, 48, and 64 are all fairly distinct.

D. Deciduoma malignum.—There is considerable irregularity in the nuclei in this growth, which was somewhat advanced, and deviations are therefore to be anticipated.



E. Combined Curve drawn from the Results shown in the preceding Four Cases of Cancer, viz., carcinoma of rectum, epithelioma of scrotum and penis, and deciduoma malignum. The three maxima about 16, 32, and 64 are unmistakeable.



Testis of *Periplaneta Americana.*

F. Curve obtained by countings made from dividing nuclei of the maiotic and premaiotic cells of the testis, in order to estimate the probable error in the far more difficult cases of cancer. It will be seen that there is some not inconsiderable variation about the two maxima of 16 and 32. This is due partly to underestimating the number of chromosomes actually present, and partly to the nuclei having in some cases been partly damaged in preparing the section.

We shall further consider this matter in the concluding part of the paper: at present we are mainly concerned with showing that there exists a striking resemblance between what we have termed the "gametoid" cells of cancer and the cells of normal reproductive tissues, and as we pass to the later phases of mitosis we find the same loop and barrel-shaped chromosomes present in both, and we have occasionally seen, during the diaster of a cancer nucleus, the late longitudinal fission in the daughter chromosomes as they diverge from each other, just as it occurs in the heterotype diaster of so many animals and plants. An inspection of the curves shows the relative frequency of the different numbers of chromosomes met with in the younger cancerous areas. Whilst, as already pointed out, the two maxima of 16 and 32 are unmistakeable, it is also obvious that amongst the generally irregular numbers two other groups occur with greater frequency than others. Thus, there is a distinct indication that nuclei containing about 24 chromosomes may be regarded as forming a distinct group, also that a second, though far less well-marked, series is characterised by containing about 64 (double the normal somatic number) chromosomes. It may be that the latter are related to the ingression of the leucocyte already described, but it is difficult at present to guess at the significance of the grouping of 24. There is no obvious indication that the nuclei with 48 chromosomes are specially common, and, in the absence of more direct evidence, it is useless to indulge in speculations that may prove to be devoid of all foundations.

In animals, as has already been stated, it invariably happens that, after the onset of the first maiotic (heterotype) mitosis, there ensues only one further nuclear division, commonly designated as the homotype, on account of its close general resemblance with a normal somatic mitosis. The principal point of constant difference lies in the retention in the former of the reduced number of chromosomes. The cells originating from this division give rise after a more or less complex series of changes of form and of the inter-relation of their constituent parts and the sexual cells without any further intervening nuclear divisions. In plants this is not the case. The cells issuing from the homotype mitosis always undergo one or (often) many subsequent divisions before some or all of the resulting units develop into sexual cells. It is therefore of interest to find in cancerous tissue that there is abundant evidence that the cells, the nuclei of which have undergone reduction, are capable of continued division, and, indeed, a great part of the tissue of the cancer is made up of such cells, which, in accordance with the terminology we have elsewhere employed, we may term post-maiotic, or "gametoid."

It will be seen that we differ from Von Hansemann in our explanation of these "hypochromatic" nuclei, regarding them as have arisen, not as the author just named believes, by a dropping-out of chromosomes from the spindle, or through some form of degeneration, but chiefly as the result of a process resembling, or identical with, that by which reduction is ordinarily effected in the tissues destined to give rise to the gametic cells. But we desire to definitely state that, in using the term "gametoid," we expressly differentiate between the cancerous cells and those of normal reproductive tissues. The relation existing between them, if any, is at present obscure; and, though we think the resemblances, which will be still further emphasised by facts we are about to describe, are very suggestive, we are far from holding the views which have been expressly or implicitly ascribed to us by other writers as to the identity of gametic with "gametoid" cells and tissues.

Finally, then, it is clear that there exist in the facts of pluripolar mitosis, on the one hand, and in amitosis on the other, a mechanism sufficient to explain all the irregular numbers encountered in a young cancer. But the irregularities, while masking, cannot conceal the far more frequently recurring numbers of chromosomes, whereby the reduced (halved) and, though far less frequently, the double, numbers become apparent. But the existence of the irregularities indicated above often renders extremely difficult the task of deciding to what category a particular departure from the normal somatic number is to be relegated.

There is a further body of evidence bearing on the resemblance between cancerous and normal reproductive tissue to be derived from a study of the so-called Plimmer's bodies of cancer.*

It was shown by one of us in 1895† that, during the prophase of the first maiotic (heterotype) division of the spermatogenetic cells of mammals, the archoplasm undergoes a peculiar and definite series of metamorphoses. In ordinary somatic or premaiotic cells, this body is seen to lie beside the nucleus as a dusky mass of protoplasm, in the centre of which are found the centrosomes. Thus, in these cells, the attraction sphere consists of the archoplasm *plus* the centrosomes (fig. 3, *b*, fig. 4, *a*).

When, however, we turn from the premaiotic or the somatic cells to the prophase of the heterotype (first maiotic) mitosis, we find these two constituents have become separated (fig. 4, *b*). The centrosomes migrate from the centre of the archoplasm, and are eventually seen to lie outside that body, and completely detached from it (fig. 4, *c*). At the same time the archoplasm itself undergoes a change, small vesicles are developed in its substance (fig. 14), and, at the close of this particular cell-generation, both vesicles and archoplasm become merged and lost in the general cytoplasm of the daughter cells.

In the prophase of the second maiotic (homotype) mitosis the same peculiar phenomena recur, and the archoplasm and the vesicles, in like manner, become lost during the later stages of this (homotype) division. In the spermatids, which result from it, the persistent centrosomes can be readily seen to be perfectly disconnected with the new archoplasm which is differentiated in these cells. The archoplasm becomes filled with minute vesicles, as in the two preceding cases, subsequently the vesicles enlarge, and they either fuse together, as in some mammals, or one usually takes the lead and grows larger than the rest, as commonly happens in the guinea-pig and in man (fig. 15). The body thus formed was originally termed the archoplasmic vesicle in 1895,‡ and it is a very conspicuous and constant feature peculiar to the sperm cells of the vertebrata, whilst it has also been encountered by various observers in animals outside that group.

When fully developed, the archoplasmic vesicle often assumes a size approximating to that of the nucleus itself, the latter being often deformed into a crescentic shape, owing to the enlargement of the vesicle that lies adjacent to it in the cell. In normal spermatids, the vesicle and its contents ultimately form the so-called "cephalic cap" of the spermatozoon (fig. 16, *a*).

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* See 'Roy. Soc. Proc.,' vol. 76 B, "On the Resemblances existing between the 'Plimmer's Bodies' of Malignant Growths and certain Normal Constituents of Reproductive Cells of Animals," by J. Bretland Farmer, J. E. S. Moore, and C. E. Walker.

† Moore, 'Internat. Monatschr. f. Anat. u. Physiol.,' 1894.

‡ Moore, *loc. cit.*

Now, the "Plimmer's bodies" are well known in the cells of many cancerous growths (fig. 17), and they are most commonly met with in the young growing portions of the tumour. They appear in the form of vesicles, and consist eventually of a fairly well-defined wall, enclosing a clear space, in which is suspended a small and densely refracting granule. They appear to occur with greater frequency in cancers of a glandular or glandular-epithelial origin.*

They lie in the cytoplasm of the cancer cells, usually in close proximity to the nucleus. They vary in size from excessively minute bodies to forms as large as the nucleus itself. The special interest attaching to the Plimmer's bodies depends on the fact that they have commonly been regarded as peculiar to cancer cells, although Honda† believes that he has occasionally encountered them in inflammatory tissue. They have, in fact, been variously interpreted. Some investigators have regarded them as parasitic organisms, more or less intimately connected with the ætiology of the disease, whilst others have seen in them a differentiation of the cancerous cell itself. Borrel‡ suggested that they might represent hypertrophied centrosomes, but the observations of Benda,§ who showed that centrosomes and Plimmer's bodies coexisted in the same cell, have rightly been held to disprove the view advanced by Borrel.

When the foregoing facts are all taken into consideration, the case originally upheld by ourselves‖ appears to be a strong one. We see no escape from the position that the Plimmer's bodies of cancer represent the archoplasmic vesicles that occur in the normal reproductive cells at the stages already indicated. And this forms an important link in the chain of similarities connecting cancerous tissue with the normal reproductive elements. But in this relation it is of interest to note that we have recently observed bodies, which appear to be closely similar to archoplasmic vesicles, to occur at apparently definite stages in the life history of certain leucocytes which are present in bone marrow.

## *General Conclusions.*

To sum up the observations already recorded in this paper, it may be seen:—

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* Greenough, 'Third Report of the Caroline Brewer Croft Cancer Com.,' Harv. Med. School, 1905.

† Honda, 'Virch. Arch.,' vol. 174.

‡ Borrel, 'Ann. Inst. Past.,' vol. 15.

§ Benda, 'Verh. deutsch. Gesellsch. f. Chir.,' 1902.

‖ 'Roy. Soc. Proc.,' vol. 76 B, pp. 230 *et seq.*

1. That a primary growth originates in the first instance as the result of a change in the nature of a number of previously functional somatic cells.

2. That the transformation may affect a considerable number of cells, and certainly continues to operate for some time.

3. That, as the result of the change, mitotic and amitotic activity is awakened, and proceeds rapidly, with a consequent increase in the mass of affected tissue.

4. That during this increase a remarkable activity prevails amongst the leucocytes, at first resembling that seen in inflammatory processes, but finally leading to the union of at any rate some of the affected cells with one or more leucocytes.

5. That in the subsequent divisions of these cells the nucleus of the leucocyte divides simultaneously with that of the cancer cell, and their chromosomes may become mingled in cleavage figure.

6. That multinucleate cells (syncytia) may arise by mitosis or by amitosis, unaccompanied with the division of the mass of protoplasm.

7. That the resulting nuclei may divide normally and mitotically, or the nuclear figures may be more or less mingled, and hence all sorts of variations in the number of chromosomes may occur. But the mode of chromosome evolution and division follows the somatic type.

8. In addition, a form of mitosis occurs, leading to nuclei with half the number of somatic chromosomes, and the phases closely accord with those observed during the heterotype (first maiotic) mitosis of animals and plants.

9. Subsequent divisions occur, in which the reduced number of chromosomes is retained, the type of division otherwise resembling that of ordinary somatic cells. These mitoses fall into the category corresponding with the post-maiotic mitoses of plants.

10. During the maiotic and post-maiotic divisions in the cancerous cells, structures are present which have been designated as Plimmer's bodies. These are common to cancerous cells and to the reproductive cells of the testis at a particular phase in their evolution. The only other cells in which structures resembling the bodies in question have been observed are possibly those forming certain of the leucocytes in bone-marrow.

It will be evident from the above summary that the change from the healthy to a cancerous development is intimately bound up with definite change in the cells affected. The onset of the change is probably to be attributed to the operation either of new stimuli upon the body cells, or to a change in the constitution of the latter. Such an alteration might originate in a variety of ways. For example, it might be ascribed to the influence of a parasite. But we have never succeeded in tracing any such cause, and it becomes necessary therefore to seek for some other explanation for the phenomena actually witnessed.

It is quite certain, in the first place, that we are dealing with the transformation of functional somatic cells into cancerous ones, and this, to our own minds, affords a complete refutation of the hypothesis as to the persistence of "embryonic rests," such as have been supposed by Cohnheim and his followers to account for the incidence of the disease.

We have drawn attention to the events that occur in connection with the invasion of the cells of the young growths by leucocytes, and, although we are fully aware that further investigations into the details of these processes are required before a final opinion can be expressed as to their true significance, the facts themselves are very suggestive.

Furthermore, the interest attaching to these fusions is not lessened by a study of the bone-marrow, in which the leucocytes can be most advantageously observed. For we have seen in this tissue all the abnormal types of nuclear and cellular division that are so highly characteristic of cancerous cells, and we have ascertained a fact of even greater importance, namely, that some of the nuclei of dividing marrow cells certainly possess less than the full complement (32) of somatic chromosomes. We would, further, lay emphasis on the occurrence, in the same preparations of bone-marrow, of other cells in which the process of mitosis was strictly somatic in character, both as regards the form and number of the chromosomes. But it is none the less certain that the other nuclei exhibit chromosomes of a remarkable form, elongated in the direction of the spindle, and strongly resembling those which are so characteristic of the heterotype mitoses of the testis or of a cancer.

Whilst it is obvious that further investigation on the cytology of bone-marrow is urgently needed, it is evident that, if it should ultimately prove that the cells which are derived from the results of fusion of a leucocyte with a tumour cell really represent the progenitors of the malignant elements themselves, a satisfactory explanation would be afforded not only of the striking nuclear character of the diseased tissues, but also of the invasive and destructive powers they undoubtedly possess. The destructive action of the leucocytes themselves on other cells of the body, especially during old age, is too well known, owing especially to the valuable researches of Metschnikoff, to call for further comment here.

Such a view of the case as is here tentatively suggested is not in conflict with the idea embodied in the term "gametoid" tissue, but rather forms an extension of it. We have, as already pointed out, from the first maintained the existence of a resemblance, extending to extraordinarily minute detail, between the "gametoid," cancerous, and the reproductive tissue, which, in the case of animals, gives rise to the gametes immediately after maiosis. But it is also now certain that there exist certain striking similarities between the leucocytic and reproductive cells which are, in themselves, highly suggestive, and this is not diminished by a consideration of the earlier phylogenetic history of wandering and reproductive cells in more primitive animals, for example, in sponges.

For the present, however, and in the absence of more complete and accurate knowledge on the evolution of the leucocytes, we may close by remarking that the various peculiar characteristics of cancerous cells find their closest analogies in the cytological processes that are exhibited in the formation of the reproductive cells, and in those maiotic phenomena that so especially distinguish them.

## DESCRIPTION OF PLATES.

### PLATE 8.

Fig. 1.—Section of the growing edge of a young Carcinoma of the Rectum.

1 *x*, 1 *y*, 1 *z*. Enlarged parts of the same drawing. The letters *c*, *d*, *e*, correspond with those on the main figure.

*a*. The portion to the right represents the normal structure of the rectum ; *b* the zone in which transmutation from healthy to cancerous tissue is proceeding.

*c*. Cell showing somatic division (see also 1 *x*).

*d*. Cells in this zone containing leucocytes (see also 1 *x* and 1 *y*).

*e*. Cell showing prophase of first maiotic (heterotype) mitosis (see also 1 *z*).

*f*. Cut portion of crypts, but belong to the zone of transformation.

*g*. Portions of the growth invading the adjacent layers.

### PLATE 9.

Fig. 2.—Section through young Epithelioma of the Penis.

2 *x*, 2 *y*, 2 *z*. Enlarged parts of the same drawing. The letters *a*, *b*, *c*, *d*, correspond with those of the main figure.

*a*. Cells showing somatic (premaiotic) divisions.

*b*. Cells showing somatic division, but with excessive number of chromosomes.

*c*. Cell showing first maiotic (heterotype) division.

*d*. Cell with leucocyte in its cytoplasm.

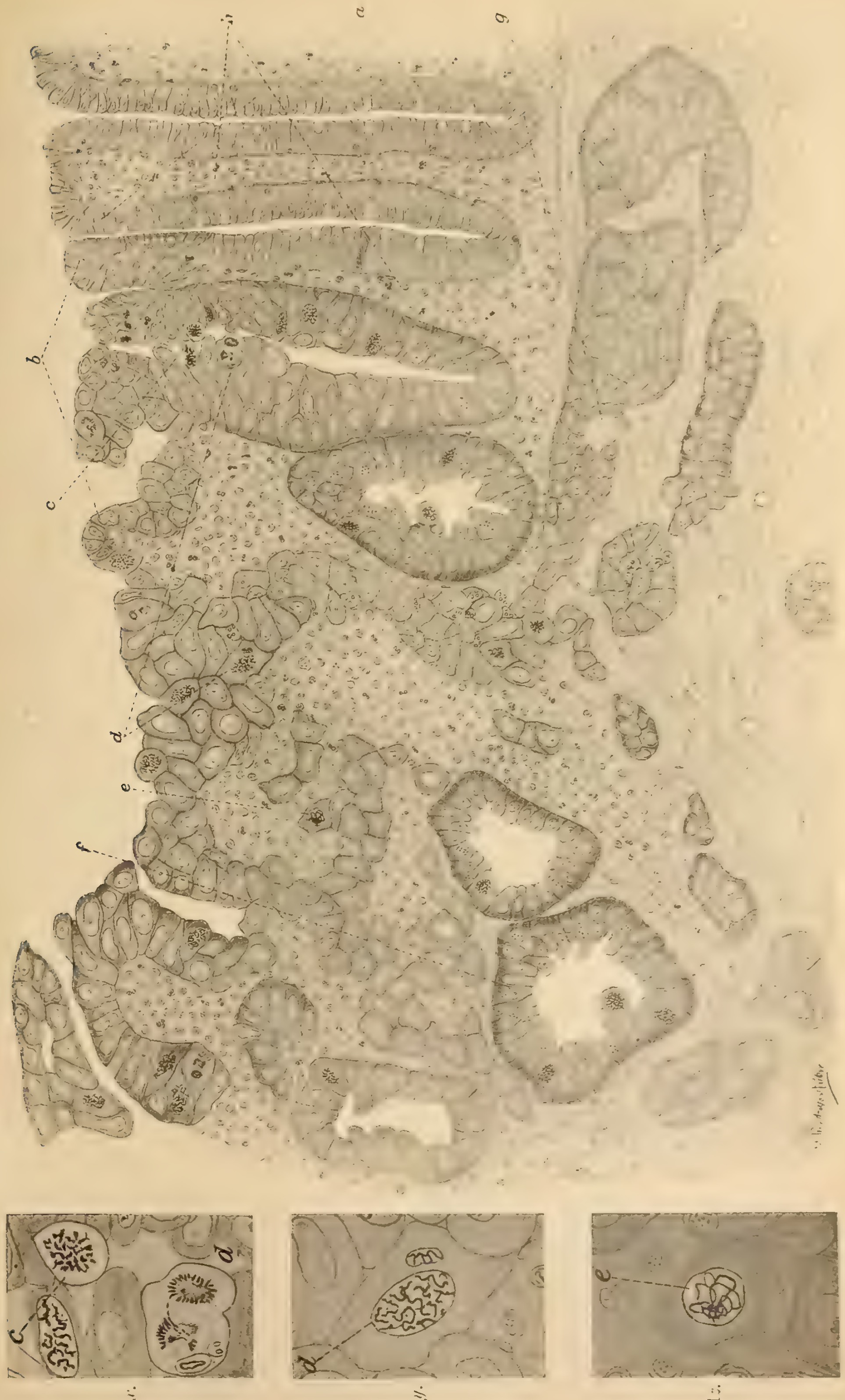
### PLATE 10.

Fig. 3.—Small Portion of the Testis of a Guinea-pig, showing (*a*) premaiotic cell dividing ; (*b*) cell in prophase of first maiotic (heterotype) division. In this it will be seen that the centrosomes are at the centre of the archoplasm.

Fig. 4.—Portion of the Testis of a Guinea-pig, showing (*a*) cells with the synaptic contraction, and the normal condition of the attraction sphere ; (*b*) late stage in the prophase of the first maiotic division, showing the centrosomes detached from the archoplasm ; (*c*) homotype prophases showing. The same dismembered condition of the attraction spheres.

Fig. 5.—Cell from the early Cancer of the Rectum given in fig. 1, showing the somatic character of division. Compare with fig. 3, *a*.

Fig. 6.—Cell from Cancer of Rectum given in fig. 1, showing the characters of the prophase of the heterotype division. Compare with fig. 4, *a*.


h
a
g
b
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f
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1 c. 1 d. 1 e.

FIG. 1.

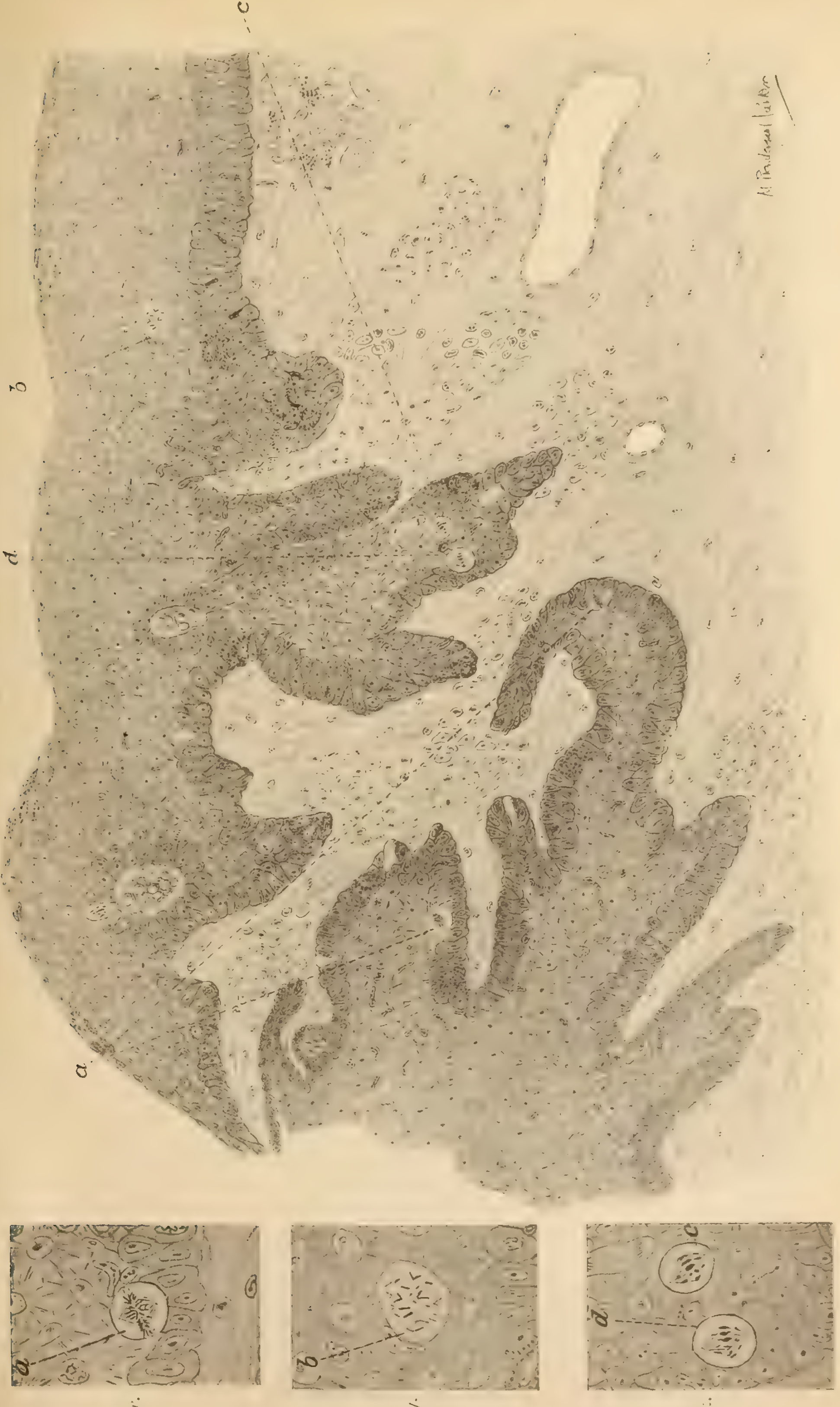


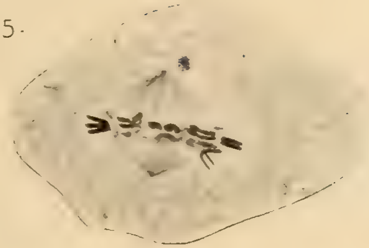
FIG. 2.

2a.

2b.

2c.

Fig. 5.



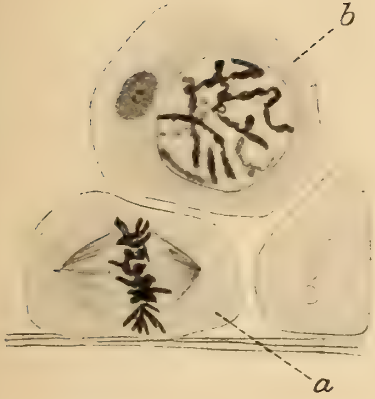


Fig. 3.

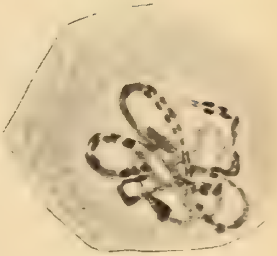


Fig. 6.

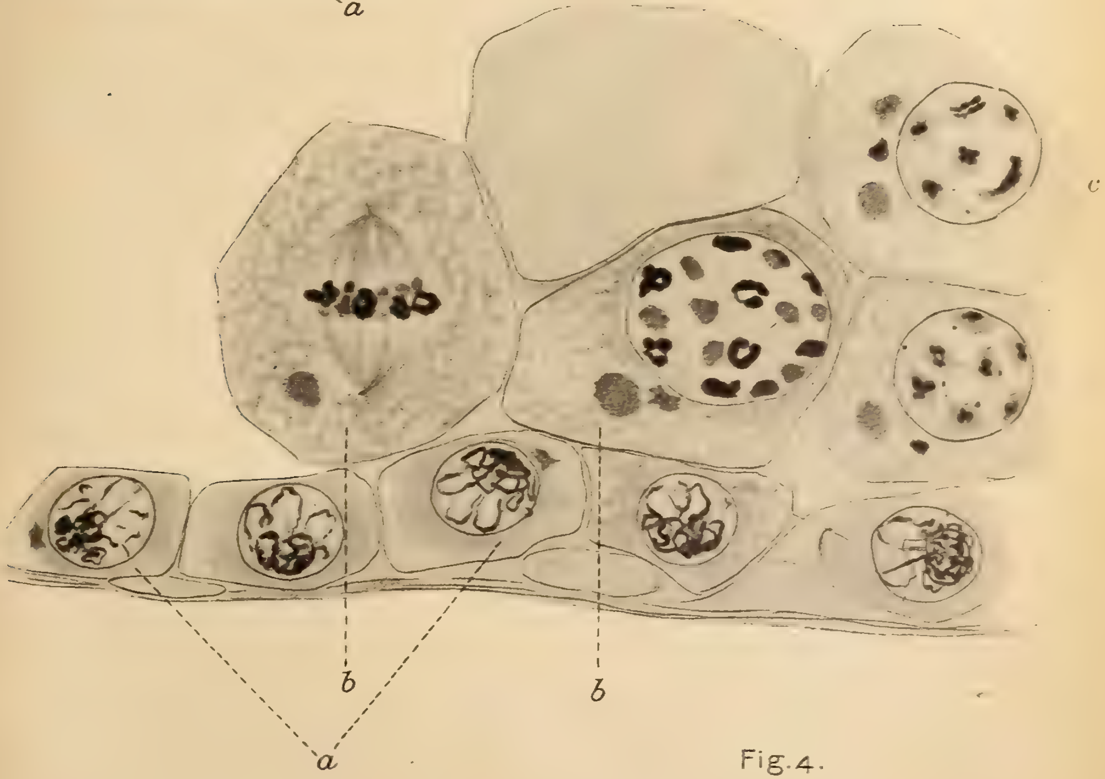


Fig. 4.

*Farmer, Moore and Walker.* *Roy. Soc. Proc., B. vol. 77, Plate* 11.

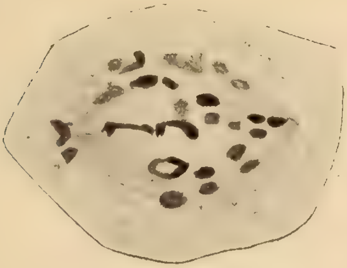


Fig. 7.

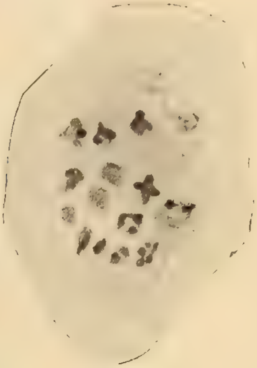


Fig. 8.

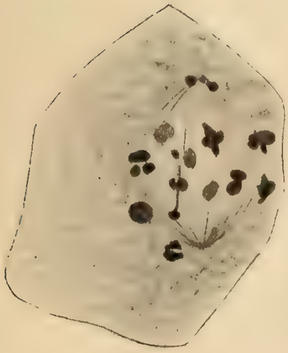


Fig. 9.

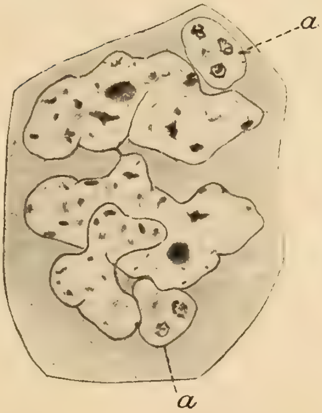


Fig. 11.

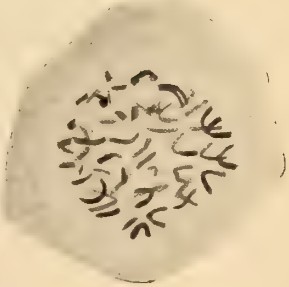


Fig. 10.

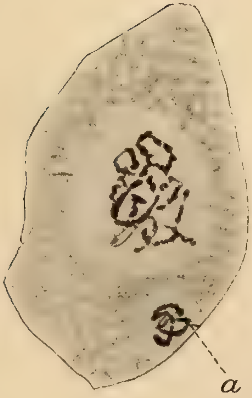


Fig. 12.

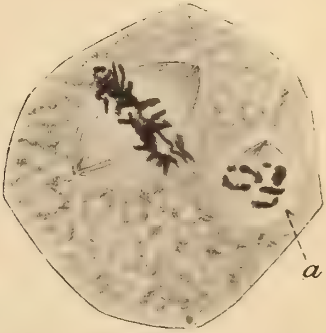


Fig. 13.

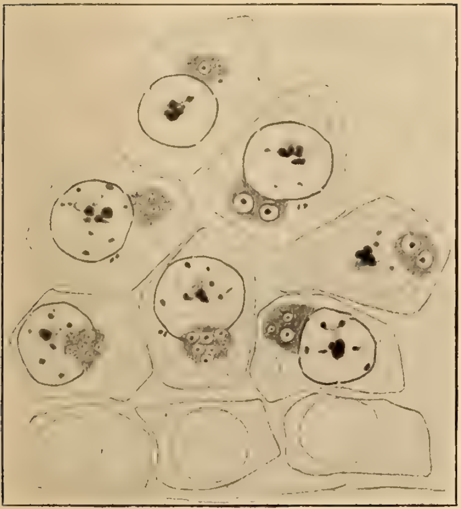


Fig. 14.

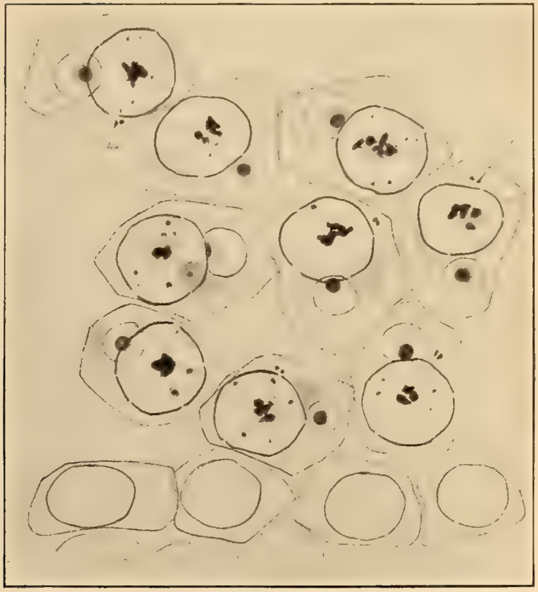


Fig 15.

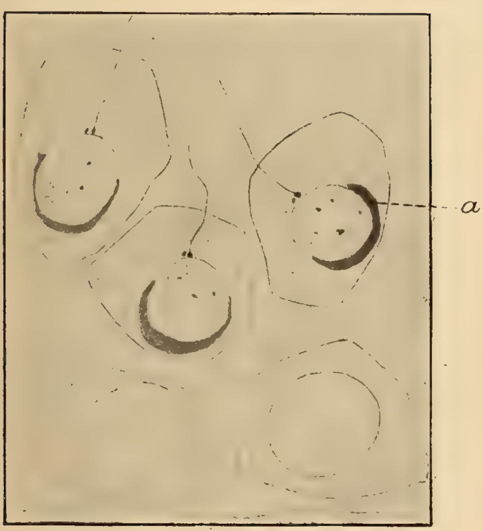


Fig. 16.

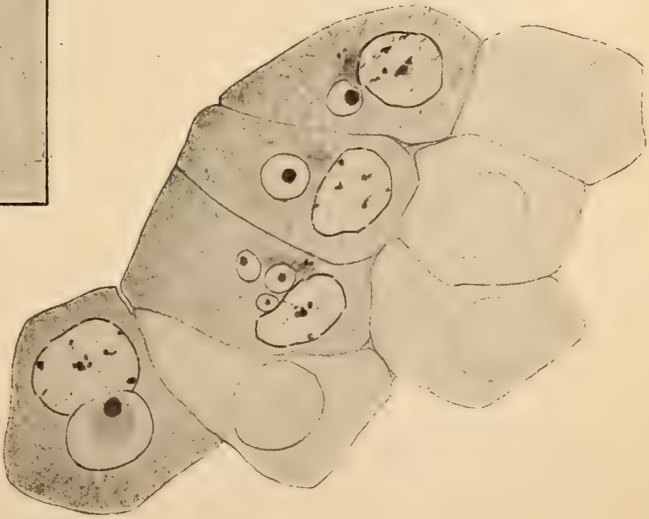


Fig. 17.

## PLATE 11.

Fig. 7.—Cell from an example of Decidua malignum, showing the later phases of the heterotype mitosis.

Fig. 8.—Similar Cell from an Epithelioma of the Tongue.

Fig. 9.—Cell from the Testis of Man, showing the later stages of the heterotype division. Compare with figs. 7 and 8.

Fig. 10.—Cell from a Cancer of the Rectum, showing the somatic or premaiotic character of the chromosomes and the large number of these elements.

Fig. 11.—Cell from an early Cancer of Rectum, showing the peculiar condition of the nucleus, which suggests amitosis; also two leucocytes (*a*) within the cytoplasm.

Fig. 12.—Cell from the same showing nucleus in the prophase of division, and also an intruded leucocyte (*a*), with its nucleus in the same phase.

Fig. 13.—Cell from Cancer of the Rectum, showing nucleus in division, and that of intruded leucocyte (*a*) in a late prophase.

## PLATE 12.

Fig. 14.—Portion of the Testis of a Guinea-pig, showing spermatids with developing archoplasmic vesicles and centrosomes.

Fig 15.—Portion of the Testis of a Guinea-pig, showing a later stage in the development of the archoplasmic vesicle. In this the origin of the tail of the spermatozoon is also seen, in connection with one of the centrosomes.

Fig. 16.—Portion of the Testis of a Guinea-pig, showing the remains of the archoplasmic vesicle becoming converted into the so-called "cephalic cap" (*a*) of the spermatozoon.

Fig. 17.—Cells from a Cancer of the Breast, showing Plimmer's bodies and the position of the centrosomes. Compare with figs. 13 and 14.

# *On the Sexuality and Development of the Ascocarp of* Humaria granulata *Quél.*

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[Plates 13—15.]

The observations of Harper (15, 16, 17) on *Sphærotheca, Erysiphe* and *Pyronema,* have clearly shown that some at least of the Ascomycetes exhibit an ordinary sexual process. It is true that attempts have been made by Dangeard (10) to refute Harper's observations, and doubt has been cast on his work by Lindau (22), Holtermann (18), and others; but the recent very convincing work of Claussen (8a) on *Boudiera,** together with the strong circumstantial evidence obtained by Barker (1 and 2) in *Monascus* and *Ryparobius,* by Miss Dale (9) in *Gymnoascus,* and by Baur (3, 4), and Darbishire (10a) in lichens, and, also, the confirmation by ourselves (7) of Harper's work on *Sphærotheca,* leave no doubt that the sexuality of the Ascomycetes is founded on a firm basis.

The earlier non-cytological observations of a number of forms, however, have shown clearly that the existence of a normal sexual process can hardly be expected in all the Ascomycetes. For example, in *Melanospora parasitica* Kihlmann (20) observed the development of the archicarp into the perithecium without the intervention of an antheridium; in *Chætomium,* Oltmanns (24) found that the antheridium was usually absent; in *Ascobolus,* the earlier observations of Woronin (25) and Janczewski (10), and the later observations of Harper (16), brought to light no definite antheridium. Again, among the lichens, according to the observations of Fünfstuck (13), in *Peltigera* and *Peltidea* the ascogonia are without trichogynes; and *Solorina saccata,* according to Baur's (4) researches, seems clearly to develop without any ordinary fertilisation. It is obviously, then, very desirable that the cytology of some member of the Ascomycetes, the ascocarp of which develops without fertilisation by an antheridium, should be carefully investigated. The form here studied is of this type and hence is of peculiar interest.

*Humaria granulata* Quél (= *Peziza granulata* Bull), a common Discomycete about 5 mm. in diameter, and of a yellow, orange or reddish tint, is found growing on the dung of various animals, especially of cow, and is most abundant during autumn and winter. The spores, apparently, normally germinate only after they have passed through the alimentary canal of the animal, for artificial cultures could not be obtained. A preliminary peptic or tryptic digestion or a combination of both seemed to have no effect on germination. Only a small number of experiments were made in this direction, for by bringing the material into the laboratory, natural cultures can sometimes be obtained, in which the fungus occurs in such abundance in appropriate stages that the necessity for artificial cultures is completely obviated.

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* The form investigated by Claussen would seem to be more correctly placed in the genus *Ascodesmis* (*vide* Fr. Cavara, 'Annales Mycologici,' vol. 3, 1905, p. 363).

The material was chiefly fixed in Fleming's weak fluid, which was allowed to act either for 24 hours or for one hour, fixation being completed in the latter case with Merkel's fluid. Either safranin, gentian violet and orange, or Benda's iron-hæmatoxylin were used for staining. The very youngest stages of the apothecia are of course quite invisible, even with a powerful hand-lens, but sections of them were secured by removing and fixing the superficial layers of the substratum on which young apothecia were just visible. The behaviour of the closely-packed nuclei of the ascogonium was best followed in sections 4 $\mu$ in thickness.

*Vegetative mycelium.*—The vegetative mycelium consists of cells which show numerous nuclei, but these, unlike those of the ascogonium, are not at all well marked, but appear generally as slightly staining homogeneous or granular bodies which sometimes show a minute nucleolar dot (Plate 13, fig. 1).

The cells of the whole vegetative mycelium and of the apothecium contain a number of fairly large spherical granules, which stain deep red with the safranin of the triple stain. In all the hyphæ of the vegetative mycelium and many of those of the ascocarp, these granules are found collected in groups on opposite sides of the transverse walls (figs. 1 and 4). These groups of granules were observed by Woronin (25) in this form and in *Ascobolus*, and by Harper (17) in *Pyronema*, but their function is unknown: Harper has suggested that they may have something to do with the passage of material from cell to cell through the wall. No reproductive organs other than the apothecia were observed in connection with this form.

## *Development of Apothecium.*

As long ago as 1866, Woronin (25) showed that the apothecium began by the development of an archicarp as a side branch of an ordinary hypha of the mycelium. He observed that the apical cell of this branch was round and very much swollen, and that, later, side branches grew up from the cells of the stalk and completely invested the apical cell. The large cell he considered to be of the nature of an egg, while one of the branches growing up from below he thought was probably of the nature of an antheridium; he was unable, however, to follow any further details of development.

*Actual Observations.*—As Woronin observed, the first beginning of the *archicarp* consists of a branch with a variable number of somewhat short cells (figs. 2A, 2B, 3). The apical cell of this row is the *ascogonium** which soon increases in size and becomes spherical, and exhibits beautiful vacuolate structure (figs. 2A, 2B). The lower cells also increase in size and both they and the ascogonium become closely filled with food-material, so that the whole archicarp has a dense and opaque appearance (figs. 4, 5). Before the ascogonium has attained its full size a number of narrow branches begin to grow out from the cells of the stalk immediately below (fig. 4). These are the first beginnings of the investing hyphæ which soon grow up and completely cover in with layers of plectenchyma the ascogonium and upper few cells of the stalk (figs. 5, 9). In cleared preparations, however, the cells of the ascogonium and of the stalk, owing to their greater density, can, for a time, be distinctly seen through the investing sheath (fig. 5).

The number of cells in the stalk is variable; there may be only a few, as is apparently the case in fig. 3, but usually a large number are to be observed, as in fig. 5. From one of the cells about the middle of the stalk a small side branch was sometimes seen which grew down into the substratum and apparently aided the stalk in absorbing nourishment.

None of the hyphæ which grow up from the stalk act in the way Woronin suggested; they are all mere vegetative investing hyphæ, *no antheridium being developed.*

The ascogonium shows a vacuolate protoplasm with a number of nuclei which are better defined than those of the vegetative cells; with the growth of this organ these nuclei become much more distinct, exhibiting a nuclear membrane and a single deeply-staining nucleolus (figs. 6, 7), but no chromatin is to be observed in the nuclear cavity. As development proceeds the nuclei increase only slightly in size but enormously in number; and the small vacuoles are replaced by one or more large ones (figs. 8, 10). At about the stage when the vegetative hyphæ completely surround the ascogonium, the wall of the latter becomes thickened and shows a distinct differentiation into two layers, the outer, thin and deeply staining, the inner, thicker and lightly staining (figs. 10, 11).

The wall between the ascogonium and the uppermost stalk cell exhibits at a young stage the usual apposed groups of granules, but at a later stage the granules apparently fuse together, for when the ascogonium has reached its full size this wall shows two large and deeply-staining masses placed opposite one another on either side of the wall (fig. 13). The masses sometimes show a central deeply-staining portion, and an outer, irregular, less dense portion (fig. 13). When the ascogonium and stalk cells become emptied these masses disappear. Besides the special accumulation on the walls a number of large granules are usually to be found scattered in the cytoplasm of the ascogonium and stalk cells (fig. 9).

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* For a discussion of this use of the terms archicarp and ascogonium, *vide infra.*

When the ascogonium has become covered in with several layers of vegetative hyphæ the ascogenous hyphæ appear as narrow, thin-walled outgrowths from the thick-walled ascogonium, and make their way through the close mass of investing hyphæ (figs. 10, 11). Into the ascogenous hyphæ there pass nuclei and cytoplasm from the ascogonium, which becomes more and more vacuolate in appearance and is finally almost completely depleted.

It is clear that the ascogonium which produces the ascogenous hyphæ has undergone no process of fertilization by male nuclei, so the development at first sight appeared to be a truly parthenogenetic one. When, however, such a case as the development of the accidium of *Phragmidium violaceum* (Blackman, 5) was considered—where, in the absence of the male cell, there is a peculiar process of fertilization by the union of a vegetative cell with the female cell—it seemed conceivable that a reduced process of a somewhat similar nature might be found in *H. granulata* also. If this were so, two possibilities presented themselves; either the ascogonium might be fertilized by the entrance of the contents of the uppermost stalk cell or of some other vegetative cell, or a fusion in pairs of the nuclei of the ascogonium might take place. As no evidence of the first possibility could be obtained, the ascogonial contents were very closely examined at various stages of development, with the result that the second hypothesis was found to be correct, and the *female nuclei were observed fusing in pairs in the ascogonium.*

These fusions are to be observed in ascogonia of various ages, sometimes when the investment of the ascogonium has only just begun, but usually at some stage between investment and the emptying of the ascogonium. There thus appears to be no definite stage of fusion for all the nuclei corresponding to that of *Pyronema*, but a gradual fusion in pairs takes place as development of the ascogenous hyphæ proceeds. The majority of fusions were observed when the ascogonium was partly emptied of its contents, as the nuclei are then not so deeply crowded as in earlier stages, and the cytoplasm does not stain so deeply.

The nuclei in most stages are so close together that it is usually impossible to distinguish from mere accidental contact the contact of nuclei which is a preliminary to fusion. In a number of cases, however, nuclei were found in pairs more or less isolated from their fellows, which were probably to be considered as on the point of fusion. The actual fusion of nuclei seems to take place very quickly, for such a stage as that of fig. 14 is rarely seen, but the nucleoli apparently remain for some time separate, for the nucleus with two nucleoli (figs. 15, 16, 17) is found more frequently. Apart from the stages of contact, which must necessarily be impossible to distinguish with certainty, more than 11 cases of actual fusion were observed, so there can be no doubt that the fusion of the ascogonial nuclei in pairs is a regular process. The size of the nuclei is not of much help in deciding whether a given nucleus is or is not the result of fusion, as three or four different sizes of nuclei may be observed in a single ascogonium (fig. 18). The nuclei apparently undergo a fairly rapid growth in size, those at the centre of the ascogonium being usually smaller, at least in the later stages, than those at the periphery.

The number of nuclei in the ascogonium varies apparently with the size of that structure, but in order to gain some idea of the number in an average ascogonium the nuclei were counted in two cases in a series of sections of an ascogonium. In the young ascogonium, of which a section is shown in fig. 7, the number 336 was determined, while the older one of fig. 10 gave 824. These countings are, of course, only approximate, as the nuclei are very crowded, and lie sometimes one above the other; also in the older ascogonium, a small number of the nuclei had already migrated, and some of those still remaining had, no doubt, already fused. The number produced by the division of the original nuclei of the ascogonium might therefore be taken as about 1000. No data were obtained as to the number of nuclei in the ascogonium at its first inception, but judging from the size of the organ at that stage and from the relatively small number of nuclei in the vegetative cells, very numerous divisions must take place. It is curious that such divisions were never observed in the ascogonium; it is probable that they are intermittent in occurrence; possibly they take place only at night.

When the nuclei pass out into the ascogenous hyphæ they show a very distinct nucleolus and are easily defined structures (fig. 11); thus the distinction of the ascogenous from the vegetative hyphæ (fig. 19) among which they ramify is rendered possible.

The ascogonium becomes finally emptied of its contents, though sometimes a few nuclei and a little cytoplasm remain behind at the periphery. Soon after the ascogonium becomes empty, the connection of the ascogenous hyphæ with it becomes obliterated, and these hyphæ appear as independent structures. This result is no doubt brought about by the pressure of the surrounding cells which leads to a slight collapse of the wall, and so to the obliteration of the cavity of the ascogenous hyphæ at their point of origin. Ultimately the whole ascogonium becomes obliterated, though it remains conspicuous as a large empty vesicle up to the time when the ascocarp first opens (fig. 31). The stalk cells also become emptied (fig. 30), and are obliterated somewhat earlier, so that after the early stages of opening no trace at all of the archicarp can be observed. It may be mentioned here that the nuclei of the uppermost stalk cell are generally more distinct than those of the ordinary vegetative hyphæ, being intermediate in structure between those and the ascogonial nuclei (figs. 11 and 12).

In the earlier stages of development the whole of the nourishment for the growth of the apothecium is supplied by the archicarp from its reserve of material, the cells of the stalk supplying the branches which arise upon them, while the ascogonium supplies the ascogenous hyphæ. In the later stages, however, a "secondary mycelium" is formed consisting of vegetative hyphæ which grow down into the substratum and absorb nourishment which is supplied to the vegetative hyphæ of the ascocarp, and so indirectly to the ascogenous hyphæ which, after the emptying of the ascogonium, are practically parasitic on the vegetative hyphæ.

The first asci are formed very early before the outer peridium is burst through (fig. 30); they arise on the ends of the ascogenous hyphæ by the peculiar process of the bending over of the apex and the fusion of the nuclei in the sub-terminal cell (figs. 20 to 25), such as has been described by Harper, Dangeard, Guillermond, and Claussen. In two cases the ascus was observed in a terminal position as described by Maire (23) and by Guillermond (14) for *Galactinia succosa*.

When the two nuclei have fused in the ascus, the fusion nucleus begins to increase in size and to show a definite chromatin substance between nucleolus and wall.

The division of the nucleus does not call for any particular comment, as it is not very favourable for investigation; the spindles are at first intranuclear and show well-marked centrosomes with radiations, but the chromosomes are too small to allow of an estimation of their number (figs. 26 to 28).

The method of spore formation in the ascus appears to be of the well-known type first described by Harper, but owing to the density of the contents of the ascus and the somewhat small size of the spores the object is not a favourable one for the study of the details of the process (fig. 29).

The paraphyses at their first appearance form a wedge-shaped mass, which appears to play a part in bursting open the peridium, as described by Harper in *Ascobolus*.

The structure of the mature apothecium is of the ordinary type; there is a definite parenchymatous peridium, a well-marked .hypothecium consisting of large cells; and the paraphyses are large and club-shaped and filled with orange granules (fig. 31).

### *General Considerations.*

It is clear that the process of fusion in pairs of the female nuclei in the ascogonium of *Humaria granulata* must be considered as a reduced sexual process which, in the absence of the antheridium, replaces the normal fertilisation by male nuclei such as we find in *Sphærotheca, Erysiphe, Pyronema,* and *Boudiera.* It renders even more untenable the most recent view of Dangeard* (10) that in the Ascomycetes as a whole there is no fertilisation in the ascogonium, but the sexual process has been shifted from that structure to the asci; for in *H. granulata* we find that even in the absence of the antheridium the process of nuclear fusion is not confined to the asci, but there is an earlier fusion in the ascogonium, which must itself be considered as the sexual process, although of a reduced type.

As stated earlier, the question of the occurrence of an ordinary sexual process in some at least of the Ascomycetes must now be considered as completely settled. Future work must decide how far the members of the group exhibit ordinary sexuality or the reduced process described above; it is possible also that some forms are truly parthenogenetic,† while there appears to be no doubt that others, as the *Exoascaceæ,* are still further reduced, the asci having a direct vegetative origin.

It can hardly be denied that the process of fusion of the female nuclei in pairs is derived by reduction from the ordinary sexual process such as we find in *Pyronema;* therefore it seems best to class such a process as a "reduced sexual process" (Blackman (5)), in which the male gamete has been replaced by another female cell (nucleus), the æcidium, just as in *Phragmidium violaceum* (5) the male cell is replaced by a vegetative cell.

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* Kuyper (21) in a recent paper, published since these observations were complete, has come to a conclusion somewhat similar to that of Dangeard. He has investigated *Monascus* and finds there only a single nuclear fusion, and that in the ascogonium, but without relation to the male nuclei. He considers *Monascus* a primitive form and that in the other Ascomycetes the fusion has been shifted to the ascus. Different results have been obtained by other workers on *Monascus*, and Kuyper's figures are not very convincing; but if there is only a single fusion, such a fusion is obviously comparable to the first fusion in *Humaria granulata* and not to the second.

† That is with potential female gametes developing without any process of cell or nuclear fusion. If there be a true alternation of cytologically distinct generations in the Ascomycetes this is not likely to occur, as true and *complete* parthenogenesis is unknown in plants possessing such an alternation.

Davis (12) has criticised such a terminology in the case of *Phragmidium*, and objects to the use of the terms fertilization or sexual process being applied to any union in which the fusion is not between the regular male and female cells. He would class these irregular processes under the head of *asexual* fusions.

It is true that a fusion in which the special sexual cells do not both take part cannot, from a *purely* morphological point of view, be a sexual process. When, however, it is considered that in some of these irregular fusions one of the sexual cells actually takes part, and also that they are of very special nature, being directly related in the phylogeny of the group to the ordinary sexual process, in fact, replacing that process in the life history, they can hardly be satisfactorily relegated to a class of *asexual* unions, where they are herded with processes most of which have not been shown to have any connection with true sexual fusions.* If on strict morphological grounds these fusions are separated from true sexual processes they should obviously be made a class apart, quite distinct from the asexual unions.†

It is doubtful, however, whether a purely morphological test of a sexual process (syngamy) is desirable when we consider that the process is essentially a physiological one and that primitively it occurs between vegetative cells (*e.g.*, *Spirogyra*, some Protozoa). Further, these irregular processes show no characters for which a parallel cannot be found in other accepted sexual processes; for in the simplest cases the fusing cells are not differentiated, and in other cases of sexuality the blood-relationship between the fusing cells (*e.g.*, lateral conjugation in *Spirogyra*, sexuality in *Basidiobolus* and many Phycomycetes) is apparently as close as in the process under discussion.

Since, then, these special processes *in themselves* have no characters which remove them from the class of sexual unions, and since they take place at a definite point in the life-cycle, and *replace in phylogeny* the ordinary sexual process, it seems proper that their relations should be exhibited in the terminology, and they should be classed as sexual processes or fertilizations, with the addition of the term "reduced," which indicates that one, or both, of the regular sexual cells has been replaced by some other cell. In the case of *Phragmidium violaceum*[*] and the "apogamous" prothallia, we may consider that there has been a sudden return in part, or as a whole, to the primitive condition where every vegetative (gametophytic) cell is a gamete.

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* Such as the fusion of nuclei in endosperm cells, and in cells which have been placed under abnormal conditions, the fusion of nuclei in the ascus, the "vegetative" cell fusions in the Florideæ, etc.

† In the present state of our knowledge the cell and nuclear unions among plants would seem to be best divided into four classes :—

(1) Cell-unions of an ordinary sexual nature :
(2) Reduced sexual processes as described above :
(3) Nuclear unions, such as are found in the teleutospore, basidium, and perhaps those of the spores of the Ustilagineæ ; these (at least in the case of some Uredineæ and probably in the other cases) are the direct result of sexual, or reduced sexual, processes which exhibit nuclear association without nuclear fusion :
(4) Asexual cell and nuclear unions, which are of doubtful or purely vegetative nature.

The third class is of very special nature, and it is not satisfactory to class them, as does Davis, with the asexual unions.

It appears, then, from the study of *H. granulata* that the female cœnogamete possesses a very striking property—*the capacity to fertilize itself.* It may be that in this capacity lies the explanation of the development without male sexual organs which seems normal for a large number of Ascomycetes; future research can alone settle this point.

Although the sexual process to be observed in *H. granulata* is, of course, morphologically reduced in relation to the normal sexual process, yet physiologically there can be little to choose between the fusion of ascogonial nuclei, which may be separated in descent by many divisions, and the ordinary sexual fusion in which, as is often the case, the antheridum and ascogonium are intimately related in origin. In fact the kinship of the fusing nuclei may very likely be closer in *Boudiera,* where sexual organs are borne in pairs on the same hypha and contain a small number of nuclei, than in *Humaria,* where the number of nuclei in the ascogonium is very great.[†] As has been suggested elsewhere (Blackman (6)), the majority of the (morphologically) normal sexual fusions in the Fungi, exhibiting as they do close-related sexual organs, are already physiologically reduced in relation to the typical (and probably primitive) exogamic sexual process. The morphological reduction found in the special fusions is thus only a small step which does not affect the physiological nature. In other words, instead of the fusion of the gametes from two gametangia borne close together and in intimate relation on the same plant, we have the abortion of the one and the fusion in pairs of the gametes of the other; put in this way it is clear that there is a morphological difference, but a physiological difference is not easily conceivable.

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[*] The case of the æcidium of another species, *P. speciosum,* in which neighbouring cells fuse in pairs, described by Christman (8), and considered by him as a simple process of conjugation of undifferentiated gametes, would seem to be much better interpreted as a reduced sexual process, in which, in the absence of the male cells (spermatia), the female gametes fuse in pairs, as in *H. granulata* (*vide* 7A).

[†] It is not asserted that the close kinship or otherwise of the fusing nuclei necessarily makes any physiological difference, but that judged by this standard the processes are essentially similar; and it is not clear that there are any other physiological factors which would differentiate the two processes.

When one considers the apparent physiological equivalence of the ordinary and the reduced sexual processes, the ease with which "self-fertilization" can be carried on in the cœnogamete, the small number of forms in which an ordinary sexual process has been observed, and the fairly large number which appear to have no antheridium, it seems not improbable that the reduced sexual process will prove to be the more common type of fertilization in the Ascomycetes.

It is obvious that the occurrence of fusions among the nuclei of the female cœnogamete itself renders still more difficult the investigation of the sexual cell of this type. The mere presence of a male organ and the observations of nuclear fusions in the female cell is now not sufficient to prove a normal fertilization; nor even is continuity between male and female organs, for the male nuclei may degenerate *in situ*, and a reduced fertilization of the *H. granulata* type may take place. To prove the existence of ordinary fertilization, evidence must be obtained for an actual migration of male elements to the female organ.

It might perhaps be suggested by some that the nuclear fusion observed by Harper in *Pyronema* were really fusions between female nuclei like those in *H. granulata.* Harper's observations on the passage of the male nuclei into the oogonium seem, however, sufficiently satisfactory to allow of this supposition being put on one side.

Dangeard's observations on *Pyronema* are very probably to be explained by the supposition that he was working on a form with a functionless antheridium. He worked with artificial cultures, while Harper used natural ones, and it has been shown by Van Tieghem (26), in a paper which seems to have been overlooked in the discussion, that *Pyronema* is very susceptible to artificial conditions. In his cultures Van Tieghem observed forms which were normal, forms which showed the ascogonium and antheridium reduced in size, and lastly forms in which the antheridium was absent, but the ascogonium developed normally. Dangeard was probably investigating a form in which the antheridium, though still present, had already become functionless; in the light of the series of forms observed by Van Tieghem, one cannot conclude with Dangeard that the antheridium is always functionless.

In such a case as *Pyronema* with a functionless antheridium a "reduced fertilization" similar to *H. granulata* is to be expected; such a process would almost certainly be overlooked unless attention was specially directed to it. The other forms lately investigated by Dangeard (10), in which either the antheridium was absent or the male nuclei degenerated, may, perhaps, also be explained by the fact that a reduced fertilization in the ascogonium was overlooked.

Exact data as to the nuclear behaviour of *Ascobolus furfuraceus* in its early stages of ascocarp development will be of special interest. It would seem likely that the nuclei fuse in pairs when they meet, as described by Harper (16), in the large ascogonial cell which gives origin to the ascogenous hyphæ. One of Harper's figures shows these nuclei in very close contact.

It is possible, also, that the parthenogenesis in other forms, in which a cœnogamete develops without the intervention of a male organ (*e.g.*, the Mucorini and Saprolegniaceæ), may be explained in the same way, by a fusion, in pairs, of the female nuclei,* and so not be a true parthenogenesis.

The fusion in the ascus still remains a most puzzling process, for which, at present, no explanation is forthcoming. That it is not a substitute for the ordinary sexual process, nor a nuclear fusion which has been shifted, in descent, from the ascogonium to the ascus, as Dangeard and Kuyper believe, is clearly shown (apart from such forms as *Pyronema, Boudiera,* etc.) by *H. granulata*, where, even in the absence of the antheridium, the fusion in the ascus is preceded by a fusion in the ascogonium. On the other hand, the curious simultaneous division of the two nuclei at the time of ascus formation—whether the ascogenous hypha bends over at the apex or whether it remains straight, as in *Galactinia succosa*—seems only to be explained as a method of ensuring that the fusing nuclei are separated in descent by at least one division. Now, such a separation, in descent, of the fusing nuclei is, as far as we know, an attribute of sexual fusions alone (though in many fusions which are accepted as sexual, the degree of relationship is very close). We have thus two closely related fusions, one of which is obviously a sexual fusion, while the other, in one character at least, partakes of a sexual nature. A satisfactory solution of the difficulty of the dual fusions can hardly be expected till we know the number of chromosomes throughout the life-history of some ascomycete.

That there is a definite alternation of generations in the life-history of Ascomycetes which possess an ascogonium seems very probable. The ordinary vegetative mycelium would appear to be the gametophyte, which bears the ascogonium, and antheridium if present, while the products of fertilization, the ascogenous hyphæ (which are parasitic on the gametophyte) and the asci, represent the sporophyte. The countings of chromosomes are, however, too few and too unsatisfactory to allow of a decision as to the cytological distinction of the two generations. It would seem, also, that there must be two reductions, as there are two fusions. The three divisions in the ascus might be expected to show at least one reduction, but Harper (17) is of the opinion that the number of chromosomes remains unaltered during these divisions. Of course, it is possible that the second fusion is of a peculiar nature and does not lead to a doubling of the chromosomes. What is obviously necessary is the discovery of a sexual ascomycete with a small number of distinct chromosomes in its nucleus, so that the number can be observed throughout the life-history.*

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* Kuyper (21) has independently made a similar suggestion in the case of the Saprolegniaceæ, and has even suggested that the figures of Davis (11) on egg development in Saprolegnia support the view of a nuclear fusion.

De Bary uses the term archicarp as practically synonymous with ascogonium. It seems much more satisfactory to use the term archicarp for the whole fertile branch, apart from the antheridium, and to confine the term ascogonium to that part of the archicarp the contents of which take part in the formation of ascogenous hyphæ, *i.e.*, the reproductive cell or cells which contain the female nuclei. It is in this sense that the terms have been used in the body of the paper. Used in this sense the term ascogonium is not necessarily confined to the cell or cells actually giving origin to the ascogenous hyphæ. In *Ascobolus furfuraceus*, for example, the whole curved fertile branch, or scolecite, is the archicarp; the central part would be the ascogonium, which is divided into a number of cells by a series of perforate septa, as Harper (16) has shown; only one of the cells of the ascogonium, however, actually gives origin to the ascogenous hyphæ, though the contents of all the ascogonial cells pass into this special cell and so into the ascogenous hyphæ. In *Melanospora parasitica*, from the observations of Kihlmann, the ascogonium is represented by one or two cells of the archicarp, though the cytological details are not known. In Pyronema, Humaria, Sphærotheca, and Erysiphe the ascogonium is a single cell, and naturally gives origin to one or more ascogenous hyphæ. In *Collema* the archicarp consists of a few small sterile cells at the base, then comes the ascogonium, which is multicellular, and above is the multicellular trichogyne; all the cells of the ascogonium appear to give origin to ascogenous hyphæ.

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* The view that there are two reduction processes is also put forward by Harper in a very important paper ("Sexual Reproduction and the Organization of the Nucleus in certain Mildews,") received while the present paper was passing through the press.

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## EXPLANATION OF PLATES.

### PLATE 13.

FIG. 1.—Portion of mycelial hypha showing the nuclei, and deeply-staining granules on the transverse wall. × 1900.

FIGS. 2A and 2B.—Two young archicarps growing up from the general mycelium : fresh preparations. × 430.

FIG. 3.—Slightly older archicarp in section. × 430.

FIG. 4.—Archicarp showing the vegetative hyphæ beginning to grow out from the cells beneath the ascogonium. The granules on the wall are clearly visible. × 620.

FIG. 5.—Young ascocarp in which the ascogonium and the sub-terminal cells of the archicarp are covered in by the vegetative hyphæ. × 430.

FIG. 6.—Section of young ascogonium showing ascogonial nuclei. × 1050.

FIG. 7.—Section of slightly older ascogonium. × 1050.

FIG. 8.—Section of ascogonium and basal cell of archicarp which have just become covered in by the vegetative hyphæ. The vegetative as well as ascogonial nuclei are clearly visible. × 1050.

### PLATE 14.

FIG. 9.—Section of ascocarp of about the age shown in fig. 5. The granules in the ascogonium and the partial emptying of the stalk cells are to be clearly seen. × 430.

FIG. 10.—Section of upper part of young ascocarp showing ascogonium partly filled with nuclei and "basal cell" below. Three ascogenous hyphæ can be traced throughout their whole length, while portions of others are visible among the vegetative plectenchyma. Nuclei are to be faintly distinguished in the "basal cell" and some of the vegetative cells. × 1050.

FIG. 11.—Section of somewhat older ascocarp showing the ascogonium and three stalk cells. The majority of the nuclei have already migrated from the ascogonium. × 620.

FIG. 12.—Section showing the nuclei in the basal cell and the cells immediately surrounding. × 1050.

FIG. 13.—Section through the lower part of the ascogonium and the upper part of the basal cell showing the curious granular masses on the transverse wall. × 1010.

FIG. 14.—Two female nuclei of the ascogonium in the process of fusion. × 2700.

FIG. 15.—A group of ascogonial nuclei with a fusion-nucleus showing nucleoli in act of fusion. × 2700.

FIG. 16.—A group of nuclei of ascogonium with a large-fusion nucleus with two nucleoli. × 2700.

## PLATE 15.

FIG. 17.—A group of nuclei from an ascogonium showing one fusion-nucleus with two nucleoli, another with nucleoli which are just fusing. × 2700.

FIG. 18.—Group of ascogonial nuclei of different sizes. × 2700.

FIG. 19.—Ascogenous hyphæ and vegetative hyphæ showing the distinction of the nuclei. × 1900.

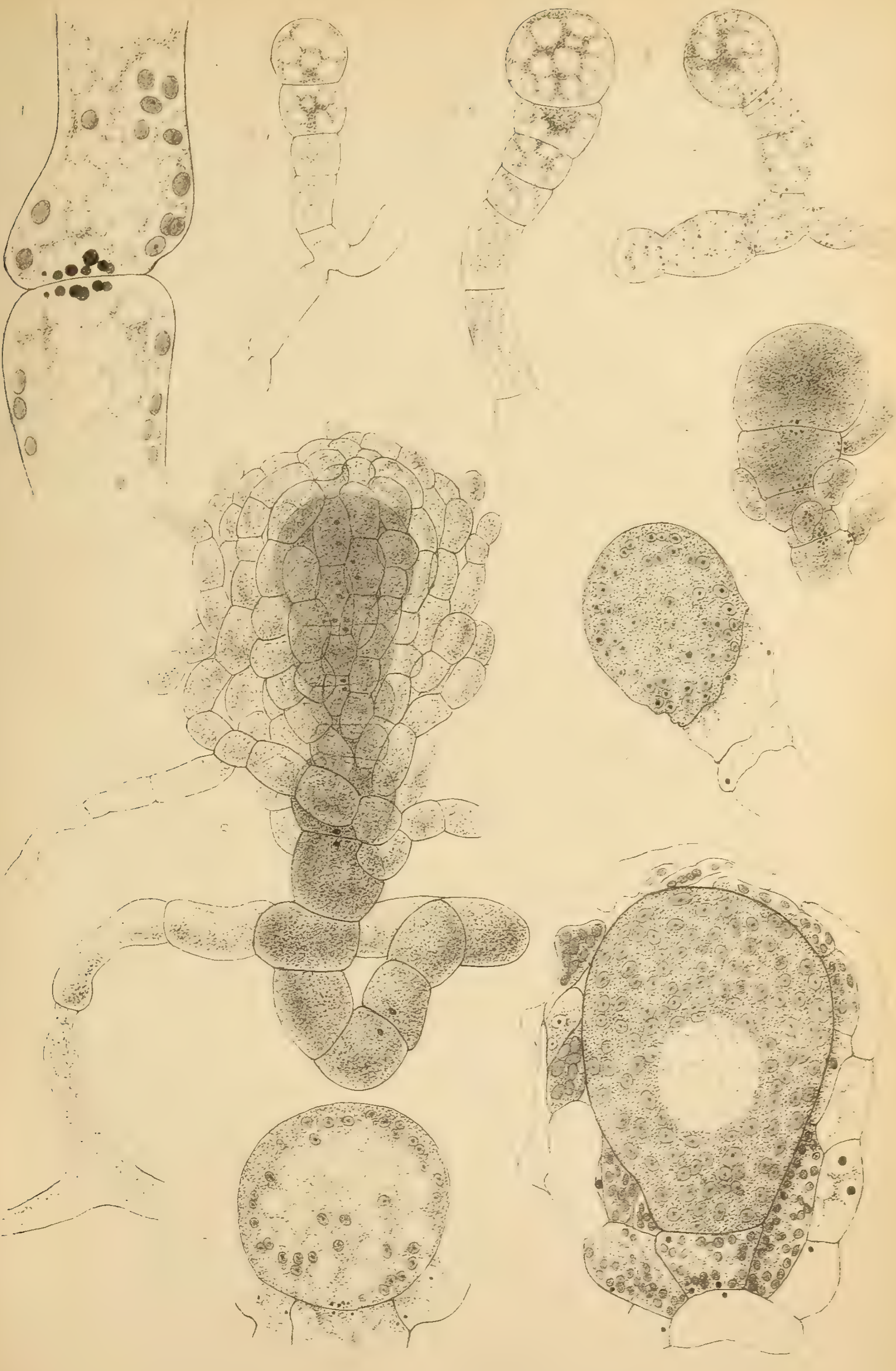
FIGS. 20—25.—Stages in the development of the ascus at ends of the ascogenous hyphæ.

FIGS. 26—28.—Three stages of the first nuclear division in the ascus. × 1900.

FIG. 29.—Spore formation in the ascus. × 1900.

FIG. 30.—Section of young ascocarp showing the wedge of paraphyses bursting through the peridium. The nearly empty ascogonium and stalk cells are visible. × 175.

FIG. 31.—Section through slightly older ascocarp when the peridium has been burst through completely, the hymenial layer definitely arranged, and a certain number of spores formed. The empty ascogonium is still visible, but the rest of the archicarp has disappeared. × 60.



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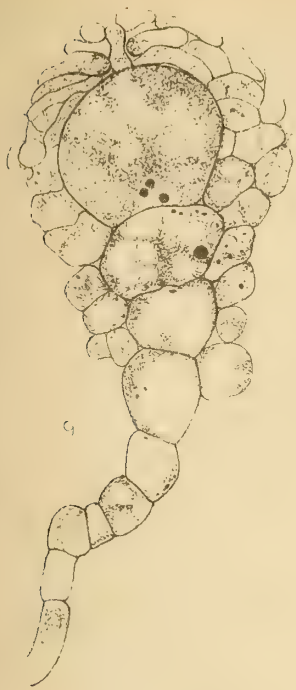
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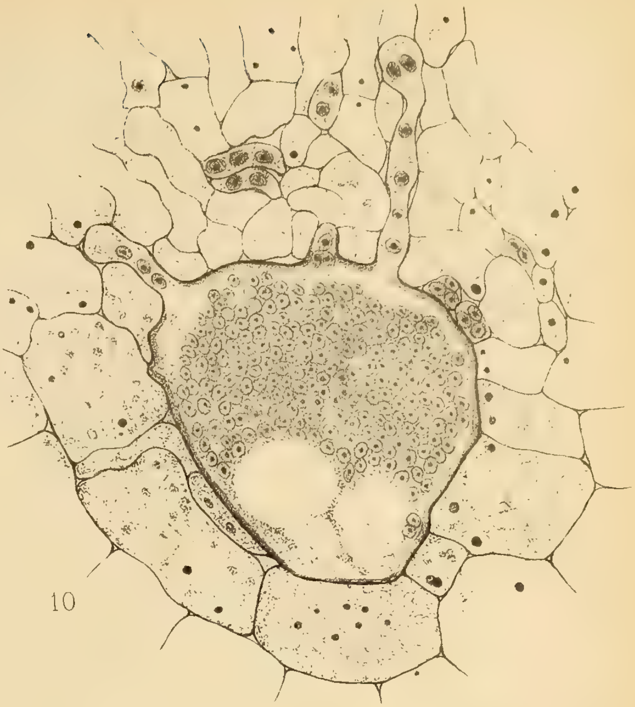
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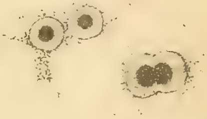
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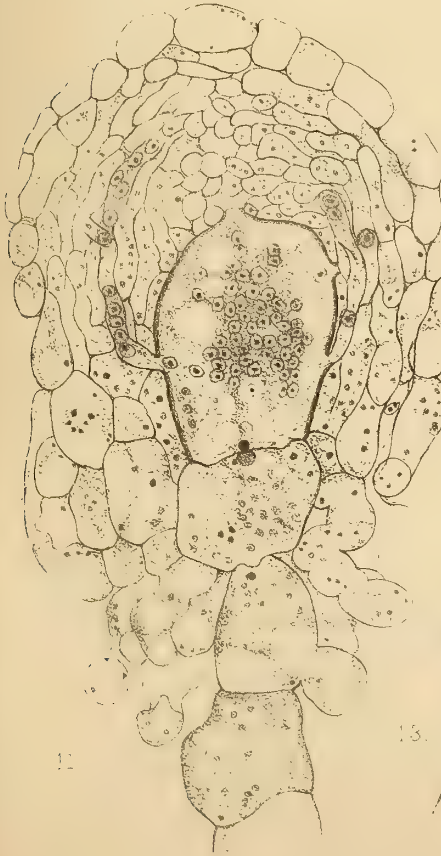
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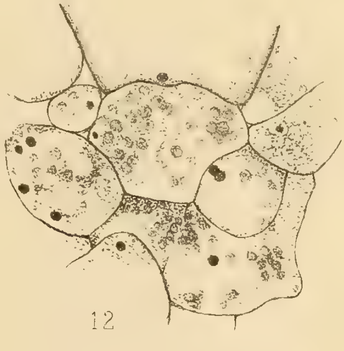
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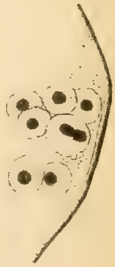
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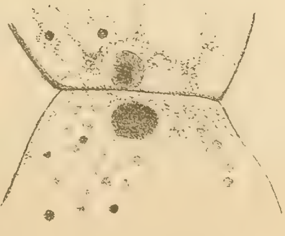
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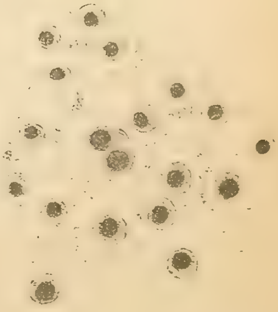
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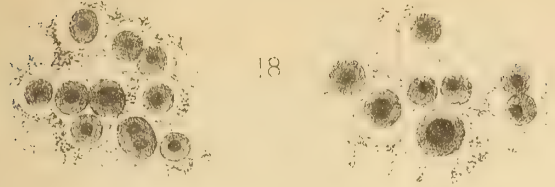
*Blackman & Fraser.* *Roy. Soc. Proc. B. vol 77 Pl. 15.*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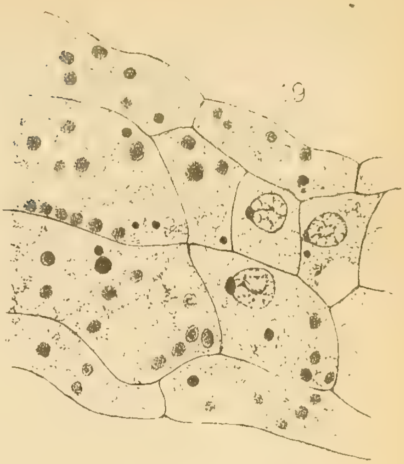
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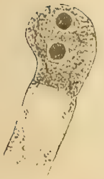
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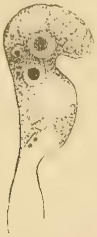
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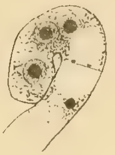
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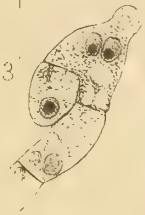
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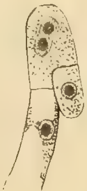

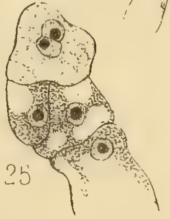
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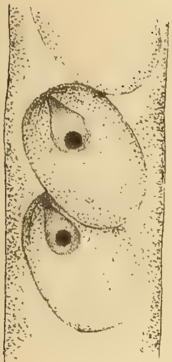
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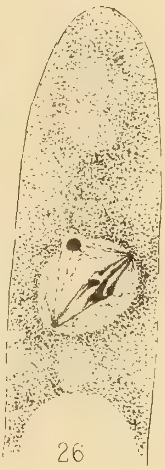
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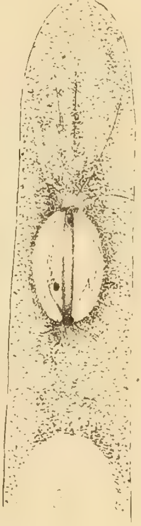

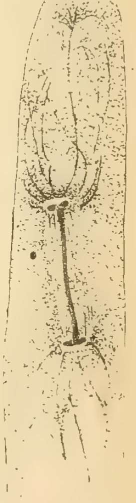
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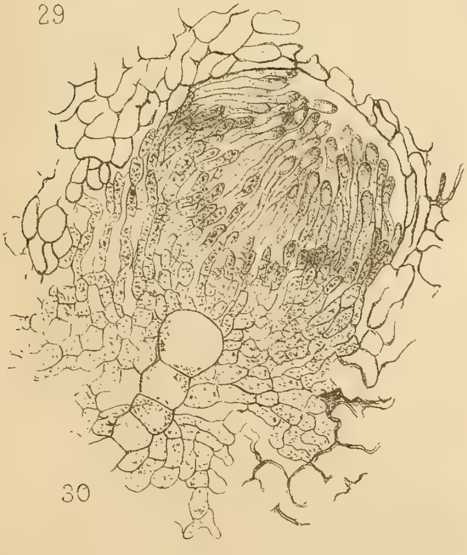
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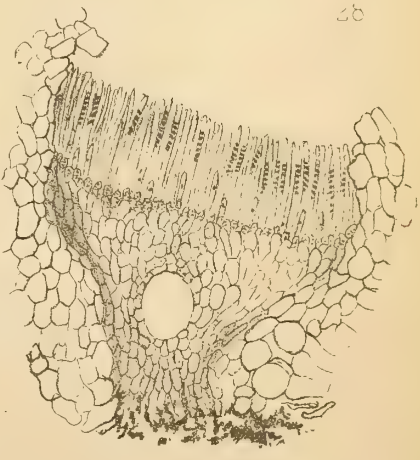
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# *A Study of the Mechanism of Carbon Assimilation in Green Plants.*

By Francis L. Usher and J. H. Priestley, B.Sc., Lecturer in Botany at University College, Bristol.

(Communicated by Professor M. W. Travers, F.R.S. Received December 16, 1905,—Read January 18, 1906.)

(From the Chemical and Botanical Departments, University College, Bristol.)

*Introduction.*—The investigation to be described in this paper has had for its object the elucidation of certain problems concerning the nature of the first stages in the assimilation of carbon from carbon dioxide by the green parts of plants; and although far from complete, it has been thought advisable to publish the results already obtained, inasmuch as the weather is likely to hinder the experimental work for some time to come.

In 1870 Baeyer put forward the hypothesis that formic aldehyde is the first product of the decomposition of carbon dioxide in the plant. This suggestion received some support from Bokorny,* who proved in 1891 that starch was formed in the dark by the green filaments of *Spirogyra* when immersed in a solution of sodium oxymethyl-sulphonate of 0·1 to 1 per cent. strength.

Bokorny's experiments are possibly open to the objection that formaldehyde condenses very readily to non-poisonous carbohydrates in presence of sulphites or bisulphites, and it has been shown by Laurent and Acton† that starch is formed in the dark from most sugars.

Quite recently, Bouilhac and Tréboux‡ have succeeded in growing plants in a very dilute solution of pure formaldehyde. Tréboux has found that *Elodea* forms starch in the dark from a 0·001-per-cent. solution of formaldehyde, and Bouilhac has shown that this is also the case with *Sinapis alba* and some *Algæ.* Their experiments bring out in a striking manner the intensely poisonous nature of even very dilute solutions of formaldehyde.

Evidence of this kind, however, is quite indirect, and on this account greater importance attaches to the results obtained by Bach,§ who for the first time demonstrated the decomposition of carbon dioxide by light outside the plant. He showed that by passing pure carbon dioxide through a 1·5-per-cent.

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* 'Berichte,' 1891, vol. 24, p. 103.

† 'Roy. Soc. Proc.,' 1890, vol. 47, p. 150.

‡ 'Flora,' 1903, p. 73.

§ 'Comptes Rendus,' 1893, vol. 116, p. 1145.

solution of uranium acetate exposed to sunlight in a glass apparatus, a precipitate consisting of a mixture of uranium peroxide with lower oxides was formed, and that the solution contained formaldehyde. Bach regarded the uranium acetate solution as playing the part of a chemical and an optical sensitiser, and considered the decomposition of the carbon dioxide to result primarily in the production of hydrogen peroxide and formaldehyde.

*Decomposition of Carbon Dioxide Outside the Plant.*—The experiments of Bach have been repeated and confirmed, both as to the production of peroxide and formaldehyde.

The amount of decomposition obtained in three weeks in bright weather was extremely small, and this appears to us to be explained by the fact that (1) as a chemical sensitiser uranium acetate is far inferior to that which exists in a green plant, inasmuch as the separated oxygen (in whatever form it may exist) is not entirely removed from the sphere of action, as in the case of the plant, but remains as a fairly insoluble peroxide which undergoes a reversible change with the other product, namely, formaldehyde; (2) as an optical sensitiser uranium acetate is inferior to chlorophyll to the extent that it possesses no absorption at all in the red, and only two faint bands between F and G.

In view of the extreme slowness of the reaction under these conditions, experiments were made with very large concentrations of carbon dioxide. Tubes of Jena glass, 40 cm. long and 8 to 10 mm. bore, were about three-quarters filled with 1·5 per cent. uranium acetate solution, and cooled in liquid air while some carbon dioxide was passed in. They were then sealed, and suspended outside a south window in bright sunshine. Within 15 minutes of warming up to the air temperature, a precipitate began to form, and in 24 hours the reaction was complete. The tubes when opened were found to contain uranium peroxide and formic acid, but no formaldehyde. The formic acid was obtained by distillation of the filtrate from the peroxide, and was characterised by (1) reduction of silver nitrate, (2) reduction of Fehling's solution, and (3) properties of lead salt.

Thus with very large concentrations of carbon dioxide, formic acid, and not formaldehyde, results.

These experiments are open to the objection that since uranium acetate is to a considerable extent hydrolysed in solution, the formaldehyde in one case and the formic acid in the other may possibly have been derived from the acetic acid present.

It has been found that uranium sulphate in a 2-per-cent. solution functions in the same way as the acetate. An experiment with the sulphate, conducted in the usual way, *i.e.*, bubbling carbon dioxide through the solution, which lasted over three weeks in very dull weather, gave uranium peroxide and formic acid. The different results obtained here from those in the case of similar experiments with the acetate, may be due to the "reduction potential" falling below the limit required for the completion of the second stage of the decomposition.

In all the foregoing experiments, except in the case of the liquid carbon dioxide tubes, blank experiments were simultaneously performed, (1) with uranium solution and carbon dioxide in the dark, and (2) with carbon dioxide free solution in the light. In neither case was any precipitate formed.

*Decomposition of Carbon Dioxide in the Plant.*—If a similar reaction, resulting in the formation of formaldehyde and a peroxide, takes place in the first stage of the absorption of carbon dioxide by the plant, it is obvious that both the initial products of decomposition must undergo a rapid change.

On account of its intensely poisonous nature, formaldehyde must be very rapidly converted into some physiologically inert substance; and the peroxide must be decomposed with evolution of gaseous oxygen, a process which follows exposure to light by an interval of one or two seconds.

The problem, then, is to ascertain the process by which oxygen is disengaged; to show the actual presence of formaldehyde localised in the neighbourhood of the chloroplasts; and to trace the steps by which the formaldehyde is polymerised.

*The Mechanism of the Evolution of Oxygen from the Green Plant.*—In the experiments relating to the decomposition of carbon dioxide outside the plant, no evolution of oxygen gas is ever observed; it remains in the system as a peroxide.

There have been conflicting statements with regard to the presence of hydrogen peroxide in plants, but even if traces are to be found, there is no evidence that it is a product of decomposition of carbon dioxide. It has indeed been shown that several organic substances, notably the organic acids, *e.g.*, oxalic, give rise to hydrogen peroxide on exposure to light, and such substances as these are of common occurrence in the leaves of plants. If, however, hydrogen peroxide is one of the first products of the photolysis of carbon dioxide, we are more directly concerned with the elimination of oxygen in the gaseous form than with the detection of the peroxide.

Hitherto those writers who have recognised the difficulty at all have suggested some method of reduction, which, of course, leads back to the starting point. It appeared much more probable that this step in the process was brought about by a catalyst, probably an enzyme. To test this, some *Elodea* was immersed in a dilute solution of hydrogen peroxide. An immediate and rapid decomposition set in, and a gas was freely evolved, which was found to be oxygen. The action proceeded as rapidly in the dark as in the light.

The following experiments were performed with the object of ascertaining the nature of the catalyst:—

(*a*) A plant was immersed in boiling water for 30 seconds and was subsequently found to be without action on hydrogen peroxide.

(*b*) After treatment with dilute solutions of iodine, mercuric chloride, and hydrogen sulphide, no action took place.

(*c*) Some *Elodea* was suspended in air charged with chloroform vapour for two hours in order to kill the protoplasm, and was then allowed to "recover" for a similar period. Rapid disengagement of oxygen took place.

(*d*) After immersion in very dilute formaldehyde solution, hydrogen peroxide was not decomposed.

These experiments seem to point to the existence of a catalysing enzyme. Several attempts to extract it by simple maceration with water or salt solution failed, and we were also unable to extract it after powdering leaves in liquid air. Following a suggestion of Dr. Horace Brown, we ultimately succeeded in obtaining it by previously drying a quantity of *Elodea*, and subsequently digesting with water at 30° for 48 hours. The enzyme was precipitated by an excess of absolute alcohol and dried.

By this process it is obtained as a light brown powder, containing diastase, whose aqueous solution energetically decomposes hydrogen peroxide, whereas ordinary malt diastase does not. Whether the enzyme is one already known, or whether it is secreted specially for the purpose of catalysing hydrogen peroxide, we cannot as yet say.

On mounting a leaf of *Elodea* in very dilute hydrogen peroxide, and examining microscopically under a high power, bubbles of gas were seen to emerge from the chloroplasts only, an observation which shows the strict localization of this enzyme to the seat of the photosynthetic process.

In regard to the distribution of this enzyme, we have examined the foliage leaves of plants belonging to 46 Natural Orders and representative of the Vascular Cryptogams and all the main groups of the Phanerogams, and have found the power of catalysing hydrogen peroxide in every case, though the energy of the decomposition varies considerably in different groups. It also occurs in etiolated leaves and in potato tubers, and, in fact, appears to be associated with amyloplasts, whether possessing chlorophyll or not.

*The Production of Formaldehyde and the Manner of its Removal.*—It has been found in the case of *Spirogyra* that starch appears in a previously starchless filament within three minutes of exposure to light, and it is probable that some sort of carbohydrate is formed much sooner than this, for it has been shown by Brown and Morris* that starch is probably not elaborated within the cell until the supply of nutriment is in excess of the cell requirements.

It would therefore seem as though the arrangement which exists in the plant for the removal of formaldehyde is at least as efficient as any external arrangement we can make to remove it in a different way, without at the same time killing the plant, and thus eliminating one of the essential factors, namely, the vitality of the protoplasm.

For this reason it is useless to look for formaldehyde in healthy assimilating leaves. It is well known that certain chemical substances possess the property of condensing formaldehyde to various carbohydrates, chiefly formose, $\alpha$-acrose, and methylenitan. It has been found by Loew† that such condensing agents are chiefly metallic oxides and acid sulphites, substances not likely to occur in plants.

Moreover, condensation by these bodies is a comparatively slow process, and quite inefficient when applied to the requirements of a plant. Nevertheless, if the condensation in the plant were due to some chemical agent stored in the neighbourhood of the chloroplast, it should still be capable of taking place when the protoplasm of the leaf is killed and its enzymes destroyed.

Some healthy green sprigs of *Elodea* were immersed in boiling water for 30 seconds, in order to kill the protoplasm and destroy the enzymes. They were then placed in water saturated with carbon dioxide and exposed to sunlight. In the course of a few hours the deep green colour of the leaves had been completely bleached, and on immersing the bleached sprigs in a solution of rosaniline decolourised with sulphurous acid, a red colour was developed.

The original green material when treated in this way exhibited no colouration. There was, therefore, some substance of an aldehydic nature present in the killed and bleached leaves which was absent in those which were alive. The sequence of events in this experiment may be described as follows:—Photolysis of carbon dioxide begins in the normal way, giving rise to hydrogen peroxide and formaldehyde. The enzymes having been destroyed, the hydrogen peroxide, instead of being catalysed in the usual manner, oxidises the chlorophyll to a colourless substance, at which point the reaction necessarily comes to an end. Meanwhile a quantity of formaldehyde, equivalent to the hydrogen peroxide required to destroy the chlorophyll, accumulates, and thenceforward the reaction is strictly reversible.

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* 'J. C. S.,' 1893, 'Trans.,' p. 632.

† 'Berichte,' 1888, p. 271.

The following experiments were performed to settle the points involved in this explanation :—It was in the first place necessary to show whether the colouration referred to above was due to formaldehyde. For this purpose some leaves, killed and bleached in carbon dioxide solution as described, were soaked for 12 hours in aniline water, and were then examined microscopically under a high power. Some leaves which had been killed and simply decolourised with hydrogen peroxide were treated in the same way.

In the first case the decolourised chloroplasts were observed to be the centres of clusters of well-defined crystals, identical in appearance with those of methylene aniline, artificially prepared from aniline water and formaldehyde. They were soluble in dilute mineral acids and also in warm alcohol, from which they crystallised in the cell on cooling. The leaves artificially decolourised with hydrogen peroxide showed no crystals.

An attempt was then made to obtain the formaldehyde outside the plant. For this purpose a large quantity of *Ulva* and *Enteromorpha* was killed and bleached in carbon dioxide solution, and subjected to steam distillation. The distillate was divided into two parts. To the larger of these was added some aniline water.

A white precipitate was formed after some time, which was collected, and heated side by side with a comparison tube containing methylene aniline. It melted, not quite sharply, three or four degrees below the pure artificially prepared substance. The other portion of the distillate was evaporated with ammonia on the water-bath, and the residue dissolved in water and treated with bromine water, gave the characteristic tetra-brom derivative of hexamethylene-tetramine.

Hence, leaves in which both protoplasm and enzymes have been killed, when placed under conditions favourable for assimilation, develop formaldehyde, until the photolytic process is brought to an end by the destruction of the chlorophyll.

It was next necessary to determine whether the condensation of the formaldehyde is due to an enzyme secreted by the chloroplast, or whether the protoplasm of the granule itself effected it. Some *Elodea* was suspended in air charged with chloroform vapour for two hours, by which means the protoplasm was killed without affecting the enzymes. It was then exposed to sunlight in saturated carbon dioxide solution. In a few hours the chlorophyll became bleached, and formaldehyde was subsequently found in the plant.

It follows from this that the protoplasm of the chloroplast is the condensing agent. The bleaching of the chlorophyll in this case is due to the fact that the enzyme, though unharmed at the commencement of the experiment, quickly becomes poisoned by the accumulating formaldehyde. In this last experiment the limit to the accumulation of formaldehyde in a plant is realised, since there is formed an amount equivalent to that amount of hydrogen peroxide which is catalysed before the enzyme ceases to act together with an amount equivalent to the hydrogen peroxide required to destroy the chlorophyll.

That a certain amount of hydrogen peroxide is catalysed when the protoplasm only is dead was shown in the following manner. Approximately equal quantities of *Elodea* were taken, one of which (A) was killed by immersion in boiling water, another (B) was suspended for two hours in air saturated with chloroform vapour to kill the protoplasm and not the enzymes, while the third (C) served as a control.

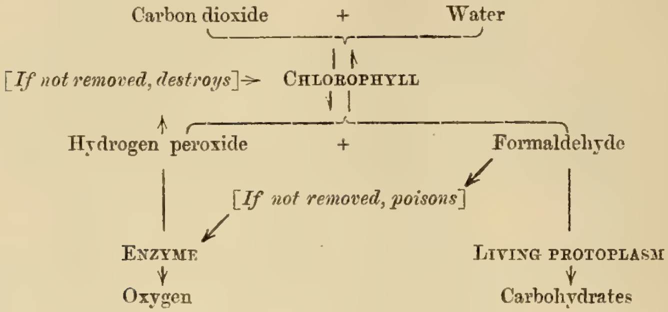
All three were placed in carbon dioxide solution under funnels with inverted test-tubes, and exposed to artificial light for 12 hours. From A there was no evolution of oxygen, from B 0·3 c.c. were given off, and from C 2·8 c.c. In the case of B, after exposure to light, no catalytic action on hydrogen peroxide could be observed, while previously vigorous decomposition had taken place.

### *Summary.*

1. The photolysis of carbon dioxide may take place outside the plant in absence of chlorophyll, provided one of the products is removed.
2. The normal products of the photolysis are hydrogen peroxide and formaldehyde, though under certain conditions formic acid may be formed.
3. In the plant the decomposition of the hydrogen peroxide is provided for by a catalysing enzyme of general occurrence.
4. The condensation of the formaldehyde is dependent on the healthy condition of the protoplasm.

There are therefore three factors essential to photosynthesis from carbon dioxide and water in the plant, they are (i) vitality of the protoplasm, (ii) presence of a catalysing enzyme, and (iii) presence of chlorophyll. If any one of these factors be interfered with, the process of photosynthesis ultimately comes to an end, through the destruction of the optical sensitiser, chlorophyll.

The relations between the various factors in this process may be diagrammatically expressed thus:—


Carbon dioxide + Water
[*If not removed, destroys*]→ CHLOROPHYLL
Hydrogen peroxide + Formaldehyde
[*If not removed, poisons*]
ENZYME
Oxygen
LIVING PROTOPLASM
Carbohydrates

In conclusion, we wish to express our indebtedness to Dr. Travers, and to Dr. Horace Brown, for their valuable suggestions and help in the course of this research.

# *A Biometrical Study of Conjugation in Paramœcium.*

By Raymond Pearl, Ph.D.

(Communicated by Professor Karl Pearson, F.R.S. Received November 15,—Read December 7, 1905.)

## (Abstract.)

1. A study of variation and correlation in conjugating and non-conjugating specimens of the common ciliate infusorian, *Paramœcium caudatum*, was undertaken for the purpose of obtaining answers to the following questions:—

*a.* Is the portion of the Paramæcium population which is in a state of conjugation at a given time differentiated in respect of type or variability or both from the non-conjugating portion of the population living in the same culture at the same time?

*b.* Is there any tendency for like to pair with like ("homogamy") in the conjugation of Paramæcium, and if so, how strong is this tendency?

The material on which this paper is based is comprised in eight series, taken from three different cultures at different times, and includes altogether the measurements of 1894 individual Paramæcia. The characters studied were length and greatest breadth of the body, length-breadth index, and the difference in length between the two individuals of a pair of conjugants. In the measuring conjugant pairs were taken quite at random, and then in each case the two undistorted non-conjugant individuals which were lying nearest in the field of view of the microscope to the conjugant pair were measured. This procedure was followed to avoid any sub-conscious bias in choosing non-conjugants.

The cultural history of the different series may be summarised as follows:—

*Series A, C, D, and E.*—These series all came from a single culture in the Zoologisches Institut, at Leipzig. This culture was set with dry hay and pond water, July 25, 1905. The dates of collection and measurement and number of individuals in each series are given in the table on p. 378.

*Series B.*—This series came from another culture at Leipzig set in the same manner as the one just mentioned. Conjugants were found on August 22, but in very small numbers. On the next day only two pairs of conjugants were found, and after that none at all. So that, all told, Series B included only 12 pairs of conjugants and 24 non-conjugants.

*Series* AA, $F_E$, *and* $F_L$.—These series included only conjugants. They were measured from material in the Zoological Laboratory of the University of Michigan, collected by Professor D. C. Worcester. They all came from a single culture set with decaying plant material and pond water. Series AA includes 200 pairs of conjugants, Series $F_E$ 70 pairs, and Series $F_L$ 77 pairs.

| Series. | Dates of measurement. | Number of conjugants measured. | Number of non-conjugants measured. |
|---|---|---|---|
| A ...... | August 15, P.M.—August 18, noon, inclusive ...... | 105 pairs | 210 |
| C ...... | „ 24, A.M.— „ 26, P.M., „ ...... | 101 „ | 202 |
| D*...... | „ 30................................................ | 16 „ | 32 |
| E ...... | September 6 ................................................ | — | 132 |

For further details regarding the measurements, culture histories, etc., the complete paper must be consulted.

2. An examination of the variation constants shows that Paramæcium is relatively slightly more variable in breadth than in length of body, though the difference is not large. For the variation in length the coefficients of variability for different series (including several other long series besides those collected in this work) are found to cluster well together about a value of 8 to 9 per cent. This is a much lower value than has been found by other workers† for variation in similar size characters in organisms with firm exoskeletons. In the characters studied Paramæcium follows the same general laws which have been shown to hold for continuous variation in higher forms.

3. It was found that conjugants are markedly differentiated from non-conjugants living in the same culture in both type and variability. This differentiation includes all the characters studied. An idea of its extent and direction may be gained by an examination of the following table. In it are given (*a*) the absolute differences between conjugants and non-conjugants in respect to the character and constant designated; (*b*) the probable errors of these absolute differences; (*c*) the relative differences defined as the percentage which the absolute difference is of the non-conjugant constant in each case. The absolute differences are taken as positive when the non-conjugant constant is greater. Only two series (A and C) are taken here as illustrations. In the complete paper similar data for other series are given in detail.

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* Series D and B (*vide infra*) include only a few individuals, because at the time they were collected no more conjugants were to be found in the cultures from which they came.

† For detailed references, see complete paper.

Table I.—Differentiation of Conjugants from Non-conjugants.

| Series. | Character. | Constant. | Absolute difference between non-conjugants and conjugants. | Relative difference between non-conjugants and conjugants. |
|---|---|---|---|---|
| | | | | Per cent. |
| A | Length ...... | Mean ...................... | 21·833 ±0·893 micron | 11·5 |
| „ | „ ...... | Standard deviation ... | 4·337 ±0·631 „ | 27·9 |
| „ | „ ...... | Coefficient of variation | 1·517 ±0·349 per cent. | 18·5 |
| „ | Breadth...... | Mean ...................... | 8·456 ±0·335 micron | 16·0 |
| „ | „ ...... | Standard deviation ... | 1·700 ±0·237 „ | 28·96 |
| „ | „ ...... | Coefficient of variation | 1·714 ±0·484 per cent. | 15·4 |
| „ | Index......... | Mean ...................... | 1·510 ±0·171 „ | 5·4 |
| „ | „ ......... | Standard deviation ... | −0·195*±0·121 „ | 7·8 |
| „ | Length and breadth | Coefficient of correlation | 0·3107±0·0526 | 52·7 |
| C | Length ...... | Mean ...................... | 33·341 ±1·098 micron | 15·9 |
| „ | „ ...... | Standard deviation ... | 6·005 ±0·777 „ | 31·4 |
| „ | „ ...... | Coefficient of variation | 1·684 ±0·398 per cent. | 18·5 |
| „ | Breadth...... | Mean ...................... | 11·050 ±0·324 micron | 20·4 |
| „ | „ ...... | Standard deviation ... | 2·491 ±0·229 „ | 42·2 |
| „ | „ ...... | Coefficient of variation | 2·984 ±0·456 per cent. | 27·4 |
| „ | Index......... | Mean ...................... | 1·416 ±0·155 „ | 5·5 |
| „ | „ ......... | Standard deviation ... | −0·152*±0·110 „ | 6·8 |
| „ | Length and breadth | Coefficient of correlation | 0·4072±0·0542 | 66·4 |

This table shows that the differentiation between conjugants and non-conjugants is strikingly large in both type and variability for all characters. The same is true for the organic correlations between length and breadth. The conjugant individuals, when compared with non-conjugants, are found to be shorter and narrower, and less variable in both length and breadth. The conjugants have a lower mean index—or, in other words, are relatively more slender—and are more variable in shape of body, as indicated both by length-breadth index and by the organic correlation between length and breadth. The conjugants have the length and breadth less highly correlated than the non-conjugants.

4. To determine whether there was any definite tendency for like to pair with like (homogamy) in the conjugation of Paramæcium, the correlation between the two members of conjugant pairs was determined for (*a*) the

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* The higher variability of the length-breadth index in the conjugants really arises from the fact that the organic correlation between length and breadth is much lower in conjugants than in non-conjugants.

same character in each member, *e.g.*, length of A with length of B (direct homogamic correlation), and (*b*) a different character in each member, *e.g.*, length of A with breadth of B (cross homogamic correlation). A part of the results for direct homogamy in respect to the character length are shown in Table II.

Table II.—Direct Assortative Pairing in the Conjugation of Paramœcium. Lengths only.

| Series. | Characters. | | Coefficient of correlation. | Number of pairs. |
|---|---|---|---|---|
| A | Length of A* | Length of B* | 0·5327±0·0333 | 105 |
| C | ,, A | ,, B | 0·7249±0·0225 | 101 |
| D | ,, A | ,, B | 0·4302±0·0972 | 16 |
| B | ,, A | ,, B | 0·7941±0·0509 | 12 |
| AA | ,, A | ,, B | 0·5882±0·0221 | 200 |

It will be seen that these coefficients are relatively very high. The only quantitative determinations of the degree of homogamy in any organism which have hitherto been made, are those for assortative mating in man, made by Pearson and his collaborators.† For the characters so far investigated in man, the coefficients of direct homogamy fall uniformly below 0·3. It is evident that we are dealing here with a much more intense assortative pairing.

For the other characters, breadth and index, the direct homogamic correlations are not so high as for length, though still relatively large. Taking the means of the results for the different series, I find for the direct homogamic correlation of breadth with breadth $r = 0·3028$, and for the correlation of index with index $r = 0·4291$.

The cross homogamic correlations (length of A with breadth of B and *vice versâ*) were found to be low in all cases. The average value of the coefficients for these cross correlations was 0·1082.

It will be noted that the homogamic correlations are in all cases positive.

5. In order to test whether the observed homogamic correlations were real or spurious, various sorts of random pairings were tried. The first question which might be raised is as to what would be the value of the coefficients measuring homogamy, provided we experimentally paired together conjugants or non-conjugants quite at random. This has been done several times for

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* A and B refer respectively to the first and second individuals of a conjugant pair to be measured.

† 'Biometrika,' vol. 1, p. 373, and vol. 2, pp. 481—498.

each series of measurements with entirely uniform results. The coefficients, measuring direct homogamy in random pairs of (*a*) conjugants; (*b*) non-conjugants; or (*c*) pairs in which one member is a conjugant and the other a non-conjugant, are uniformly zero within the limits of the probable errors.

It might be maintained that since at different points in the culture and at different times the environment no doubt differs slightly, there would be a corresponding differentiation of the Paramœcia in each local unit of the culture. Then even though the pairing were really quite at random in each locality, yet if the records for several such localities were mixed, a spurious homogamic correlation would arise. Now it is clear that if the observed homogamic correlations were spurious and due to this "local differentiation" factor, we ought to get sensibly as high values if we consider as a pair the two individuals lying nearest in the field of view to each pair of conjugants measured. Such pairs will have come from the same environment and have been killed at the same instant as the actually conjugated pairs. The homogamic correlations for these pairs of individuals, lying nearest to conjugant pairs, have been determined and found to be sensibly zero, taking into account their probable errors. This I take to prove that the observed homogamic correlations are not to be explained as due to local differentiation within the culture.

6. The existence of a high degree of assortative pairing with respect to length can be shown in another way. We may consider as a character of conjugant pairs the difference in length between the two members. Forming frequency distributions of this difference for (*a*) conjugated pairs; (*b*) random pairs of conjugants; and (*c*) random pairs of non-conjugants (the two individuals lying nearest each conjugant pair measured), I find that the mean difference in microns is from two to three times greater in the random pairs—whether of conjugants or non-conjugants—than it is in the actually conjugated pairs. In more than 60 per cent. of the conjugated pairs, the two members differ in length by less than 8 microns. There is a much greater "scatter" of the variates about the mean in the case of the random pairs as compared with the conjugated pairs. These results for the difference distributions are, of course, what would be expected if there is a high degree of homogamy in respect to length.

7. In order to determine whether the observed homogamic correlations are due to a real "assorting" in the pairing, or, on the other hand, arise from some process of equalisation in size occurring after union has taken place, the following test was made:—Two series of conjugants were measured and the homogamic correlations calculated. In the first of these series were included only pairs which were known, on the basis of their nuclear condition,

to be in very early stages of the conjugation process. In the second series were included only pairs which, on the same basis of nuclear condition, were known to be about to separate shortly. Now if the observed homogamy is a result of an equalisation of the members of the pairs in size during the conjugation process itself, clearly the second of these two series ought to show the higher homogamic correlations, because the individual pairs are in late stages, and we should expect to get the maximum effect of any equalisation which had occurred. As a matter of fact the coefficients of direct homogamy in length are $0{\cdot}6797 \pm 0{\cdot}0307$ for the "early" series and $0{\cdot}6212 \pm 0{\cdot}0334$ for the "late" series. The coefficients are sensibly equal for the two, with what advantage there is in favour of the "early" rather than the "late" series. From this it is concluded that the observed homogamy is not due to an equalisation in size during the conjugation process itself.

8. From evidence which is discussed in detail in the complete paper, it appears probable that the observed homogamic correlations arise as the result of a process, the essential factors in which may be outlined as follows: (*a*) At the periods of conjugation the individuals which are to conjugate are in a so-called "miscible" condition in which the oral surfaces are adhesive; (*b*) as a result of the action of the currents, produced by the oral groove cilia, two individuals which by chance happen to be swimming parallel and close to one another are drawn together and their oral surfaces adhere in whole or in part; (*c*) the extreme anterior ends of the oral grooves *firmly* adhere to one another first; (*d*) if the two individuals are so nearly the same size that the mouths approximately coincide when the anterior ends are together, firm union occurs at the mouth regions, and definite conjugation follows; (*e*) if, on the other hand, the mouths do not approximately coincide, the individuals separate again or die and no conjugation results; (*f*) the homogamic correlations arise then as a result of the necessity for the mouths of the two individuals to come together (or "fit") when the extreme anterior ends are united. Individuals in which the distances from the anterior end to the mouth are approximately equal, will not be greatly different in total length, and hence their lengths will be correlated. The direct homogamic correlations for breadth and index, and the cross homogamic correlations, are held on this view to have arisen because of the organic correlation between length and breadth in the individual.

9. In concluding, some of the theoretical bearings of the work are discussed. It is pointed out that if the results obtained in this work with reference to the existence of a differentiated "conjugant type" are true, they

are of considerable significance in connection with current views regarding the theoretical relation of the Protozoa to evolutionary problems. The results show clearly that, so far as the present material is concerned at least, (*a*) there is a differentiated "conjugant type" of Paramæcium; and (*b*) that this "conjugant type" is relatively fixed and constant under varying environmental conditions, as compared with the type of the general population in fission generations. If the individual Paramæcia of a given race must conform to a definite and relatively fixed morphological type every time they conjugate, what they may acquire during fission generations is clearly of no particular account to the evolutionary history of the race in the long run. Real evolutionary progress will depend on changes in the "conjugant type," just as in the Metazoa real evolutionary progress depends on changes in the germ cells rather than in the soma.

Attention is called to the significance which a high degree of homogamy, whenever it exists, must have as a factor in divergent evolution. A high degree of homogamy furnishes at once the means whereby a new variety or species may be differentiated from a parent species, though both continue to live together in the same area or environment. Because, if like mates with like, with any considerable regularity or uniformity, whenever individuals appear showing deviations from the general population, those which have on the whole like deviations will tend to mate together rather than with individuals unlike themselves belonging to the parent stock. Hence, the variations will not be "swamped by intercrossing" before natural selection has an opportunity to act on them. Hitherto, the existence of a high degree of homogamy has not been demonstrated quantitatively in any organism living in a state of nature. The results of the present work show that not only is such a degree of homogamy possible under natural conditions, but that it actually exists in one case of what must be considered as the simplest forerunner of the sexual processes of higher organisms, namely, the conjugation of a protozoon.

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# *The Growth of the Oocyte in Antedon: a Morphological Study in the Cell-Metabolism.*

By Gilbert C. Chubb, D.Sc., Assistant to the Jodrell Professor of Zoology, University College, London.

(Communicated by Professor E. H. Starling, F.R.S. Received December 20, 1905,—Read January 18, 1906.)

(Abstract.)

In this paper I have endeavoured to interpret in terms of the cell-metabolism the structural changes exhibited by the growing ovarian egg of *Antedon bifida*, Penant. The following conclusions are based entirely on the study of this single type. It will be my endeavour in subsequent papers to ascertain how far these conclusions are applicable to the eggs of other types and to the cell in general.

*The Yolk Nucleus.*—Throughout the growth of the oocyte the nucleolus intermittently discharges groups of deeply basophile spherules into the cytoplasm. In the young oocyte these "nucleolar spherules" remain unchanged, slowly accumulating in the cytoplasm, where they form small groups near the germinal vesicle. In slightly older oocytes the increased fluidity of the cytoplasm which results from the progressive accumulation of metaplastic material in preparation for yolk formation, causes the discharged nucleolar substance to lose its spherular form, and to diffuse on to the neighbouring cytoplasm. The more deeply staining area of the cytoplasm to which this diffusion gives rise is the yolk nucleus. As the cytoplasm becomes still more fluid, the yolk nucleus assumes the regular form of a concavo-convex lens embracing the spherical germinal vesicle.

During yolk formation the yolk nucleus passes to the periphery of the egg, a migration also shared by the "peripheral spherules." The actual increase in size which the peripherally placed yolk nucleus shows during the earlier stages of yolk formation is speedily masked by the great superficial extension and thinning out on the surface of the egg which this structure undergoes during yolk formation, and which is the necessary result of the great increase in bulk and surface area accompanying this process.

As yolk formation progresses the yolk nucleus gradually loses its staining capacity, and, before this process is completed, has entirely disappeared from view. At no time, however, does the yolk nucleus show any indication of a granular disintegration, nor does it show any spatial relation to yolk

formation. Yolk formation takes place in this area of the cytoplasm exactly as elsewhere.

The clear area often accompanying the yolk nucleus, usually on its outer surface, is an artifact, and results from the inability of the acid fixing reagent to coagulate the metaplastic substances with which the cytoplasm, and especially the yolk nuclear area of the cytoplasm, is loaded.

The yolk nucleus, therefore, is simply a region of the cytoplasm on to which waste material discharged from the nucleolus has diffused. The period at which this diffusion occurs, as also all subsequent changes in the form and position of the yolk nucleus, are determined by the progressive change in the physical consistency of the cytoplasm which precedes and accompanies yolk formation. Thus the yolk nucleus, like the nucleolar material discharged subsequent to its formation, takes no part in the cell-processes.

*The Nucleolus.*—The spherical form of the nucleolus, a form in which minimal surface area is associated with maximal bulk, stands in striking contrast to the subdivided condition of the chromatin, and indicates the absence of any reaction between the caryolymph and the nucleolar substance at the surface of the nucleolus; the latter structure passively growing by the deposition of material on its surface from solution in the caryolymph.

This nucleolar material consists of two substances: the one acidophile and extending throughout the nucleolus, the other deeply basophile and borne by the acidophile ground substance, to which its presence imparts a considerably firmer consistency.

Nucleolar activity, which proceeds from within outwards, results in the breakdown of the basophile constituent; the products of this process forming an irregular zone between the firm, as yet unaltered, basophile cortical region and the acidophile internal region of the nucleolus. When the internal pressure reaches a certain point the cortical layer is ruptured, and the accumulated products are discharged into the caryolymph, partly as deeply basophile spherules, partly as a clear fluid. The spherular form of the discharged material is determined by the fluid character of the surrounding caryolymph, and when the accumulated products of nucleolar activity happen to lie within a vacuole in the nucleolus, then they here also possess a spherical form. There is no indication that the acidophile ground substance of the nucleolus is itself discharged.

During the growth of the oocyte the amount of the deeply basophile nucleolar substance varies greatly, but the growth of the nucleolus nevertheless shows a strict correlation with that of the egg, and is due to the steady accumulation of the acidophile ground substance.

On the completion of yolk formation all indication of nucleolar activity abruptly ceases. The supply of the basophile constituent of the nucleolus also ceases, but the acidophile constituent continues to be deposited on to the nucleolar surface, where, no longer being incorporated by the now inactive nucleolus, it forms lens-shaped accumulations.

The selective action shown by the nucleolar material, together with the strict correlation the growth of this structure shows to that of the egg, excludes the possibility of this substance being derived directly from a source outside the cell. The fact that the ground substance continues to be deposited on to the nucleolus after the completion of all cytoplasmic activity, together with the close relation this structure shows to the chromatin in earlier stages, points to the latter as its source. The failure of the supply of the basophile constituent on the completion of yolk formation indicates its origin from the cytoplasm.

The steady growth of the nucleolus is not due to the accumulation of waste material, but to an increased production of the "ground substance" by the chromatin in order to cope with the ever-increasing production of waste material by the cytoplasm of the growing egg. From the nucleolus this waste material, now presumably inert and harmless, is discharged into the cytoplasm, where it slowly dissolves away.

*The Chromatin.*—With the increase in metabolism which accompanies the growth of the oocyte there is a corresponding finer subdivision, and more equal distribution of the chromatin. This endeavour to increase the facilities for reaction with the surrounding caryolymph indicates that the chromatin obtains its food material by active incorporation instead of passively by deposition. The absence of any reciprocal action on the part of the food material of the chromatin indicates for this substance a source outside the cell. The chromatin is, therefore, the cell-structure with which the raw food material supplied by the parent organism first comes into relation.

During the gradual accumulation of metaplastic material in the cytoplasm which marks the period preceding yolk formation, the chromatin is sparsely scattered and relatively faintly stained. With commencing yolk formation the staining capacity, and hence also the quantity visible, of the chromatin rapidly increases, and at the same time basophile droplets appear on the chromatin threads and increase in number and size during yolk formation.

The elaborated food material is passed on from the chromatin, in part to the nucleolus, but mainly to the cytoplasm. During the period preceding yolk formation, and which is marked by the progressive accumulation of metaplastic material in preparation for this process, the avidity with which the cytoplasm takes up the products of chromatin activity causes the latter structure to appear faintly stained. With the commencement of yolk formation this avidity is greatly reduced and the products of chromatin activity, now being produced in excess of the requirements of the cytoplasm, accumulate on the chromatin threads, causing the increase in the staining capacity of the latter and the formation of the basophile droplets.

*The Cytoplasm.*—Throughout the whole growth period of the oocyte there is a gradual accumulation in the cytoplasm of metaplastic material in preparation for yolk formation. This accumulation is accompanied by an increase in the basophile staining capacity, and fluid consistency, of the cytoplasm. The process of yolk formation, that is the actual appearance of the definitive yolk spherules, is unaccompanied by increased nuclear or nucleolar activity, and consists simply in the rapid and automatic conversion of the accumulated material into a form sufficiently stable to survive the period of quiesence which succeeds the completion of the egg's growth.

*The "Germinal Vesicle."*—Form of the Nucleus.—The oocyte constitutes an osmotic system, of which the cell wall forms an outer, the nuclear membrane an inner, semipermeable membrane. The accumulation in the cytoplasm of soluble substances in preparation for yolk formation causes an increase in the osmotic intensity at the outer membrane, and a corresponding decrease of that at the inner. The loss of turgescence which thus results permits of the assumption of an irregular form during fixation.

Size of the nucleus and subdivision of the chromatin.—The large size of the germinal vesicle, as also the fine subdivision of the chromatin, are due to the limitations which the care of the specific qualities imposes on the adaptive capacity of the chromatin; both these conditions being merely the expression of a more economic use of this material. The subdivision of the chromatin increases the area of contact with the caryolymph, and the increased area of the nuclear membrane through which diffusion takes place permits of a greater concentration of nutritive material within the nucleus. Up to the expansion due to the formation of the definitive yolk spherules, therefore, the size of the germinal vesicle shows a close relation to that of the egg, and is determined when equilibrium is established between the rate of diffusion of nutritive substances through the nuclear membrane on the one hand, and the requirements of the chromatin on the other.

# *On the Inheritance of Coat Colour in Horses.*

By C. C. Hurst.

(Communicated by W. Bateson, F.R.S. Received November 4,—Read December 7, 1905,—Publication deferred by request of author, December, 1905.—Additional note received February 1, 1906.)

Recent experiments by the writer have demonstrated the Mendelian inheritance of coat colour in rabbits.* These results suggested that the inheritance of coat colour in horses might also be in accordance with the Mendelian principles. In the case of horses, however, the ordinary method of direct experiment was impracticable, so that recourse was had to the valuable series of records contained in 'Weatherby's General Stud Book of Race Horses.' This work consists of 20 volumes, containing fairly complete records of the age, colour, sex, and parentage of British thoroughbreds from the earliest accounts down to the end of 1904.

In the modern volumes, at all events, these records have been carefully registered, and with the allowance of a small margin of error, may be safely accepted as scientific evidence, as the results of this paper show.

Professor Karl Pearson has already made use of this material in his paper "On the Inheritance of Coat Colour in Horses,"† but his statistical methods did not disclose any intrinsic differences in the heredity of the several colour-types. More recently, referring to the inheritance of eye and coat colour, Professor Pearson remarks that:—"Before we leave the cases above it is just worth reiterating that nothing corresponding to Mendel's principles appears in these characters for horses, dogs, and men."

In regard to thoroughbred horses, he adds:—"It is the same with every coat colour taken, its relative constancy depends largely on the extent to which it has appeared in the ancestry, and one by one black, bay, chestnut, grey must be dismissed by the Mendelian as neither 'recessive' nor 'dominant,' but as marking 'permanent and incorrigible mongrels.'"‡

A careful examination of the 'Stud Book' records so far fails to give any support to Professor Pearson's statement: on the contrary, the records show clearly, for instance, that bay and brown are Mendelian dominants to chestnut which is recessive.* For example, the bay and brown sires *St. Simon*, *St. Serf*, *Galopin*, *Ladas*, *Merry Hampton*, and *Cabin Boy*, mated with numerous chestnut mares, gave 370 foals, all of which were bays or browns. The following table gives the individual numbers:—

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* 'Journ. Linn. Soc. (Zool.),' vol. 29, pp. 283—324 (1905).

† 'Phil. Trans.,' A, vol. 195, pp. 79—150 (1900).

‡ 'Biometrika,' vol. 2, pp. 214, 215 (1903).

Table I (DD × RR).

Pure Dominant Bay and Brown Sires, Mated with Recessive Chestnut Mares.

| Sires. | Bay and brown foals. | Chestnut foals. | Total. |
|---|---|---|---|
| St. Simon ............... | 96 | 0 | 96 |
| St. Serf .................. | 83 | 0 | 83 |
| Galopin.................. | 62 | 0 | 62 |
| Ladas...................... | 56 | 0 | 56 |
| Merry Hampton ...... | 44 | 0 | 44 |
| Cabin Boy............... | 29 | 0 | 29 |
| Totals ......... | 370 | 0 | 370 |

The above numbers not only show the Mendelian dominance of bay and brown over chestnut, but they also prove that the individual sires concerned are homozygous bays and browns giving off no chestnut gametes.

It may be noted that all these sires had bay or brown parents and grandparents except *St. Serf*, which had a chestnut grandparent.

Further records show that other bay and brown sires are heterozygous, giving off chestnut gametes on the average in equal numbers, in accordance with the Mendelian expectation.

For instance, the bay and brown sires *Royal Hampton*, *Donovan*, *St. Angelo*, *Isinglass*, *Orvieto*, *Ayrshire*, *Florizel II*, *Pioneer*, *Isonomy*, *Melton*, *Wisdom*, and *Rose Window*, mated with numerous chestnut mares, gave 702 foals, of which 355 were bays and browns and 347 were chestnuts. These numbers are close to the Mendelian expectation of equality.

The following table gives the individual numbers:—

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* In the recent volumes, about 95 per cent. of the colours registered are bay, brown, or chestnut, the small remainder consisting of black, grey, roan, and other colours.

Table II (DR × RR).

Impure Dominant Bay and Brown Sires, containing Chestnut, Mated with Recessive Chestnut Mares.

| Sires. | Bay and brown foals. | Chestnut foals. | Total. |
|---|---|---|---|
| Royal Hampton ...... | 44 | 44 | 88 |
| Donovan ............... | 47 | 33 | 80 |
| St. Angelo............... | 37 | 41 | 78 |
| Isinglass ............... | 28 | 34 | 62 |
| Orvieto .................. | 32 | 30 | 62 |
| Ayrshire ............... | 31 | 29 | 60 |
| Florizel II............... | 34 | 25 | 59 |
| Pioneer .................. | 26 | 26 | 52 |
| Isonomy ............... | 25 | 24 | 49 |
| Melton .................. | 20 | 23 | 43 |
| Wisdom.................. | 18 | 23 | 41 |
| Rose Window ......... | 13 | 15 | 28 |
| Totals ......... | 355 | 347 | 702 |

Of the above heterozygous bay and brown sires containing chestnut, *Royal Hampton, St. Angelo, Isinglass, Orvieto, Isonomy, Melton,* and *Wisdom* had each a chestnut parent; *Donovan, Pioneer,* and *Rose Window* had both parents bay or brown, with a chestnut grandparent; *Ayrshire* had all the parents and grandparents bay or brown, with a chestnut great-grandparent; while *Florizel II* had all the parents, grandparents and great-grandparents bay or brown, with a chestnut great-great-grandparent. It will be noted that, notwithstanding these differences in ancestry, all are heterozygous, giving off chestnut gametes in about equal numbers, in accordance with the Mendelian expectation.

Further records show that the recessive chestnuts, variously extracted from bays and browns, as a rule breed true when mated together without reversion to their bay and brown ancestors, in accordance with the Mendelian conception of gametic purity.

For example, 100 chestnut sires, variously extracted, mated with about 600 chestnut mares of various extractions, gave 1104 foals, of which 1095 are recorded as chestnuts and 9 as bays or browns. That is to say, more than 99 per cent. of foals bred from chestnut parents are recorded as chestnuts, while less than 1 per cent. are recorded as exceptions to the rule. The apparent exceptions to the rule of chestnuts breeding true might have some significance if they were more numerous, but they are too inconsiderable to be of any certain value, and may simply represent breeders' or printers' errors.

In a work of such a magnitude and character as the 'General Stud Book,' it is satisfactory to find that in a given case, tested by Mendel's law, 1 per cent. covers the margin of error, and the result reflects great credit on all those concerned in the compilation of the records.

The following table gives the individual numbers of 30 of the chestnut sires:—

Table III (RR × RR).

Chestnut Sires, of Various Extractions, Mated with Chestnut Mares of Various Extractions.

| Sires. | Bay and brown foals. | Chestnut foals. | Total. |
|---|---|---|---|
| Amphion | 0 | 56 | 56 |
| Kendal | 1 | 49 | 50 |
| Wiseman | 1 | 48 | 49 |
| Chittabob | 0 | 45 | 45 |
| Despair | 0 | 45 | 45 |
| Juggler | 1 | 41 | 42 |
| Otterburn | 1 | 40 | 41 |
| Bend Or | 0 | 40 | 40 |
| Ocean Wave | 1 | 38 | 39 |
| Deuce of Clubs | 0 | 36 | 36 |
| Orion | 0 | 35 | 35 |
| Sainfoin | 0 | 28 | 28 |
| Southampton | 1 | 27 | 28 |
| Esterling | 0 | 27 | 27 |
| Necromancer | 0 | 27 | 27 |
| Satiety | 0 | 25 | 25 |
| Saraband | 0 | 24 | 24 |
| Llanthony | 1 | 23 | 24 |
| Hagioscope | 0 | 21 | 21 |
| Timothy | 0 | 19 | 19 |
| Hazlehatch | 0 | 18 | 18 |
| Lowland Chief | 0 | 18 | 18 |
| Lord Lorne | 0 | 17 | 17 |
| Aperse | 0 | 16 | 16 |
| Albert Victor | 0 | 16 | 16 |
| Dan Dancer | 0 | 16 | 16 |
| Gold | 0 | 15 | 15 |
| Peter | 0 | 13 | 13 |
| Friar's Balsam | 0 | 13 | 13 |
| Young Woodpecker | 0 | 11 | 11 |
| Monte Cristo | 0 | 11 | 11 |
| Seventy other chestnut sires | 2 | 237 | 239 |
| Totals | 9 | 1095 | 1104 |

It being established that chestnut is a Mendelian recessive, the question arises, What is the critical feature which distinguishes chestnut from bay and brown? An examination of many horses leaves little doubt that the chestnut is distinguished by the absence of black "points" (mane, tail, and legs) always present in bay and brown. In all these types the colour of the coat presents a considerable range of tint. The differentiating pair of Mendelian characters is thus apparently the presence or absence of black pigment in the "points."

### *Summary.*

In modern thoroughbred horses chestnut colour is a Mendelian recessive to bay and brown, which are dominant characters.

The consideration of other colours being excluded, bays and browns are of two kinds:—(*a*) Those that when mated with chestnuts will give *no* chestnut offspring. (*b*) Those that when mated with chestnuts will give, on an average, half their offspring chestnuts and the remainder bays or browns. Similarly, the recessive chestnuts, variously extracted from the dominant bays and browns, breed true, as a rule, when mated together, without reversion to their bay or brown ancestors. To this rule, 9 exceptions were found in 1104 cases, and it is not impossible that these may be due to errors in the records.

### *Note added January* 31, 1906.

In the paper read on January 18, Professor Weldon disputes these conclusions, while admitting that, contrary to Professor Pearson's statement, chestnuts breed true within a very small percentage of error. Since my paper was read, a fresh tabulation has been made of a sample including more than 2500 offspring recorded from chestnut mares, taken without selection.

Omitting greys, two sires of doubtful identity and sires with less than 10 foals each, it appears that—

30 RR sires give 455 R + 3D,
25 DD „ 450 D + 4R,
54 DR „ 496 D + 507 R.

This evidence, with that previously collected and with Professor Weldon's fresh evidence, points to about 1 per cent. of exceptions in the records of both the chestnuts and the pure dominants. Professor Weldon's argument is based entirely on the alleged existence of exceptions. The Stud Book is remarkably accurate, but there is a sufficient margin of demonstrable error in the returns to make it possible that the few exceptions which cannot be eliminated are due rather to mistake than to any physiological peculiarity in the animals concerned. As a matter of fact, a sensible proportion of the original records are afterwards corrected, either in the Stud Book itself or in the Racing Calendar. In illustration of this, it may be mentioned that

*Ben Battle*, a sire recorded in the Stud Book as chestnut, appears in the Racing Calendar several times as bay or brown, and Mr. G. H. Verrall, who has kindly given help in this matter, writes that it is practically certain that *Ben Battle* never ran as a chestnut.

Several alleged chestnuts, ascribed to *Hackler*, *Wolf's Crag*, and other pure dominant sires can be similarly corrected. Among the residual exceptions are some which were born dead or died unnamed. Very few indeed can be proved to have appeared in public uncorrected. Other errors probably arise through an incorrect return of the sire's name. For instance, a certain sire appears to give a total of 43 dominants and three chestnuts from chestnut mares; a scrutiny revealed that two of these chestnuts (which happen to be among the four mentioned above) were from one chestnut mare. Moreover, the same mare is credited with a *bay* foal by a *chestnut* sire. These three exceptions occur in four consecutive returns from one stud. Considering the extreme rarity of any exception, the coincidence seems to point to inaccuracy in the returns of the breeder in question. In Professor Weldon's tabulation such a sire would be ranked as a DR, and the 43 bays and browns he gave would go to create the excess of dominants which Professor Weldon found to result from the mating RR × DR—an excess obviously due to the inclusion of such cases in that category. It is, of course, not impossible that genuine exceptions do occur. They must, however, be exceedingly rare in any case, and I am disposed to doubt whether the returns made to the Stud Book have the extreme precision which would be required to establish such occurrences.

Finally, it would appear that the distinct properties of chestnuts must be ascribed to segregation rather than to ancestry, seeing that their behaviour in heredity is entirely different from that of bays and browns, though their ancestral composition may for several generations have been the same.

This analysis was undertaken without any knowledge of previous work on similar lines, but several papers stating more or less concordant conclusions have since been discovered. The memoir by Crampe,[*] mentioned by Professor Weldon in the discussion on his paper of January 18, gives, as I now find, extensive tables drawn from German sources, showing that within a small margin of error chestnut (*fuchs*) breeds true. Of the exceptions, several, as he shows, are probably mistakes, and the rest he regards as dubious. Wilckens,[†] as the result of a similar analysis on a large scale, found 24 recorded exceptions to the purity of "*fuchs*" per 1000 matings. The absolute purity of chestnuts, however bred, is asserted by Mr. Wilfrid

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[*] Landw., J. B., 1888, vol. 17, especially p. 828.

[†] *Ibid.*, p. 575.

Scawen Blunt* for the Kehailan strain of Arabs. The same assertion is also made for the horses called "*rote*" (? red chestnut) in the Jutland breed by Jensen.† Finally, we have a general statement by Hayes,‡ applicable to all kinds of horses, that in the vast majority of cases a foal from a chestnut dam by a chestnut sire is of a chestnut colour.

The existence of the two kinds of dominants, and the conclusions based thereon have not, so far as I know, been previously recognised.

## *Note on the Offspring of Thoroughbred Chestnut Mares.*

By W. F. R. Weldon, F.R.S.

(Received January 15,—Read January 18, 1906.)

The colours of English thoroughbred race-horses, as recorded in Weatherby's General Stud-Book, are grouped under the six main categories—Grey, Roan, Chestnut, Bay, Brown, Black; but each category includes a considerable range of colour, and intermediates occur with quite sensible frequency. At the meeting of the Royal Society, held on December 7 last, the suggestion was made that the relation between the most important colours, Chestnut, Bay, and Brown, might be expressed by a simple Mendelian formula—Bay or Brown being regarded as determined by one Mendelian unit, which was "dominant" to the "recessive" Chestnut. In discussing this suggestion, I made certain statements, which I promised to justify as soon as possible, by offering the data, on which they were based, for publication. The object of the present note is partly to fulfil my promise, and partly to call attention to certain facts which must be considered in the attempt to apply any Mendelian formula whatever to the inheritance of coat colour in race-horses.

I have taken from Weatherby's Stud-Book a fairly complete record of all the foals produced in England by Chestnut mares during the period (rather more than eight years) covered by vols. 18 and 19. All cases of doubtful paternity have been excluded, and I have not tried to obtain external evidence in the few cases where the colour of sire or of foal is omitted. With these limitations, I believe the following statements are substantially complete and accurate.

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* 'The Nineteenth Century and After,' 1906, January, p. 63.

† 'Deut. Pferdezucht.,' I, Hft. 11, 1904. (Original not seen; abstract in 'J. B. Landw. Pfl. u. Tierzüchtung,' 1905, II, p. 273.)

‡ 'Points of the Horse' (3rd ed.), 1904, p. 326.

The Chestnut mares recorded produced, in the period dealt with, 5643 foals by 726 sires, the colours of the sires being as follows :—

| Colour of sire. | No. of sires. | Colour of sire. | No. of sires. |
|---|---|---|---|
| Ch. | 214 | Br. or Bl. | 10 |
| Ch. or Ro. | 2 | Bl. | 8 |
| Ch. or Bl. | 1 | Gr. | 3 |
| B. or Ch. | 1 | Ro. or Gr. | 1 |
| B. | 340 | Br. or Gr. | 1 |
| B. or Br. | 24 | Gr. or Bl. | 1 |
| Br. | 120 | | |
| | | | 726 |

It will be seen that 41 of these sires are intermediate between some two of the accepted colour-categories.

Omitting the Roan or Grey sires, the foals produced are shown in the following table :—

Offspring of Chestnut Mares.

| Colour of sire. | Colour and frequency of foals. | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Ch. | Int. Ch. | B. | B. or Br. | Br. | Br. or Bl. | Bl. | Totals. |
| Ch. .................. | 1568 | — | 18 | 2 | 3 | 1 | 2 | 1594 |
| B.......................... | 1118 | 7 | 1449 | 89 | 132 | 4 | 8 | 2807 |
| B. or Br............. | 44 | 1 | 85 | 9 | 21 | 2 | 1 | 163 |
| Br. .................. | 233 | 2 | 439 | 44 | 146 | 13 | 14 | 891 |
| Br. or Bl. ......... | 22 | — | 5 | 1 | 5 | 1 | 3 | 37 |
| Bl. .................. | 13 | — | 28 | 5 | 11 | 2 | 8 | 67 |
| | 2996 | 10 | 2024 | 150 | 318 | 23 | 36 | 5559 |

1. *Chestnuts Mated Together.*—It is clear that Chestnut cannot be regarded as a Mendelian recessive, since Bay, Brown, Black, and their intermediates, may be produced by the union of two Chestnut individuals. The number of foals not Chestnut, resulting from such unions, is 26, or about 1 in every 60 of the whole number observed. They were produced by 21 out of the 214 sires recorded, or about 1 sire in every 10 produced at least 1 foal which was not Chestnut. Since the mean number of foals per sire is only 1594/214 = 7·4 through the period observed, the most probable number of

sires giving foals of colour other than Chestnut, if each sire were capable of giving 1 such foal in 60, would be 7·4/60 × 214, or about 26, the standard deviation of the expectation being nearly 4·6; so that the result obtained is not inconsistent with the view that any Chestnut horse is capable of producing, by union with a Chestnut mare, a small proportion of foals of some colour not Chestnut. If each sire had produced but a single foal, we should clearly have to expect that only 1 sire in 60 would produce a foal of abnormal colour. Among the sires recorded, 56 have produced only a single foal each, and of these foals one is Bay or Brown.

On the other hand the Chestnut sires, *Amphion* and *Despair*, have produced 115 foals, all Chestnut, while *Amalfi*, *Brag*, and *Dog Rose* have together produced 36 Chestnuts and seven of other colours during the period examined.

We may conclude: (1) That the mean chance of obtaining a foal which is not Chestnut from two Chestnut parents is about 1/60; and (2) that this chance is not the same for all sires; it is probably less than 1/60 for *Amphion*, *Despair*, and their like, and much greater for such sires as *Amalfi*, *Brag*, and *Dog Rose*. This variability in the power of transmitting colours other than their own, possessed by Chestnut sires, is precisely what Galton's theory of dominance would lead us to expect; it is, however, a difficulty in the way of any Mendelian theory involving a reasonably small number of elements.

2. *Chestnuts Mated with Bays and Browns.*—On the view that Bay is a Mendelian "dominant" to Chestnut, Bay sires should be sharply divided into two series: those which are "hybrid," or of constitution "DR," giving 50 per cent. of Chestnut foals and 50 per cent. of Bays by the unions considered, and those which are "pure dominants," giving only Bay offspring. Any Bay horse with one Chestnut parent must be "hybrid" on this view; but it does not follow because both parents are Bay that a Bay colt is a pure dominant. In the table below, those Bay sires which had *either* one Chestnut parent, *or* were known to have produced a Chestnut foal, are classed as "DR"; while a Bay whose parents were both of some colour other than Chestnut was classed as DD unless I could find a record that it had produced a Chestnut foal.

Among the 65 sires classed as DD, no less than 37 have produced only one foal each during the period dealt with. Some of the sires are so young that their whole progeny from Chestnut mares is too small to afford a test of their real nature; others are so old that the labour of testing them by reference to previous volumes of the Stud-Book is great, and involves risk of error. For such reasons as these, the number of sires classed as DD is certainly rather above than below the truth. In spite of this, however, the proportion of Chestnut foals produced by the sires called DR is too small for any simple Mendelian theory. The simple Mendelian expectation is half, or 1235 Chestnuts, the standard deviation being $\sqrt{2470/4}$, or about 24·8. The observed deviation is $-77$, or more than 3·1 times the standard deviation, and a reference to the Tables of the Probability Integral shows that the chance of getting a deviation so great as this is only 0·0009, or a little less than one in a thousand.

Chestnut Mares × Bay Horses.

| Sire. | Colour and frequency of foals. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Ch. | Ch. Ro. | Ch. Bl. | Ch. B. | B. | B. Br. | Br. | Br. Bl. | Bl. | Totals. |
| "DR" ......... | 1118 | 1 | 1 | 4 | 1141 | 68 | 88 | 4 | 5 | 2430 |
| "DD" .. ...... | — | — | — | 1 | 308 | 21 | 44 | — | 3 | 377 |
| | 1118 | 1 | 1 | 5 | 1449 | 89 | 132 | 4 | 8 | 2807 |

The matings with *Brown* horses, treated in the same way, give similar results.

Chestnut Mares × Brown Horses.

| Sire. | Colour and frequency of foals. | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Ch. | Ch. B. | Ch. Br. | B. | B. Br. | Br. | Br. Bl. | Bl. | Totals. |
| "DR" ............ | 233 | — | — | 246 | 25 | 72 | 9 | 9 | 594 |
| "DD" ............ | — | 1 | 1 | 193 | 19 | 74 | 4 | 5 | 297 |
| | 233 | 1 | 1 | 439 | 44 | 146 | 13 | 14 | 891 |

Here the simple Mendelian expectation is 297 Chestnuts, so that the observed deviation from the required result is $-64$, the standard deviation of the expected result being $\sqrt{594/4} = 12{\cdot}2$, or the observed result is even more improbable than that given by Bay sires.

If we attempt to consider some more complex Mendelian theory of the relation between Chestnut and Bay or Brown, we must base our hypothesis of the number of gametic determinants involved upon some separation of sires into groups, those of each group giving a definite proportion of Chestnut foals when mated with Chestnut mares. My observations do not at present decide this point; but through the period I have dealt with no such separation of sires into groups seems possible. Taking only the Bay or Brown sires which have given at least 20 foals in the period covered by my records, I find—

| Sire. | Foals. | |
|---|---|---|
| | Ch. | Not. |
| Oberon | 1 | 42 |
| Blue Green | 1 | 28 |
| Minting | 2 | 31 |
| Veracity | 2 | 29 |

at one end of the series and at the other such sires as *Rightaway* (21 Ch. + 10 Not), or *Queen's Birthday* (13 Ch. + 3 Not), which offer a transition to such Chestnut sires as *Amalfi.*

The detailed discussion of individual sires, and of the proportion of Chestnuts yielded by each, must be postponed until the work of extracting the records from the Stud-Book has been completed. In the meantime, however, the following points seem clear:—

1. No simple Mendelian view of the relation between Chestnut, Bay, and Brown, regarding Chestnut as a simple recessive, can be maintained.

2. The chance of getting a Chestnut foal from a Chestnut mare is not constant for sires of any colour whatever, and there is no indication that sires of any colour can be sorted into groups such that those in each group will give Chestnut foals in a Mendelian proportion when mated with Chestnut mares.

3. These points, together with the values for parental and grand-parental correlations already given by Professor Pearson* make it probable that the facts of inheritance of coat colour in horses can be expressed in terms of the hypothesis outlined by Mr. Galton in 1872,† and developed by him in his subsequent writings.

I hope to discuss the application of Mr. Galton's hypothesis to these data before long; but the extraction of pedigrees from the Stud-Book is an operation which takes time, and I have felt bound to offer this imperfect statement in justification of what I said in the discussion to which I have referred, without waiting until my work should be completed.

* 'Phil. Trans.,' A, vol. 195.

† 'Roy. Soc. Proc.,' No. 136, pp. 394—402.

# *Chemical Action of Bacillus lactis aerogenes* (Escherich) *on Glucose and Mannitol: Production of 2 : 3-Butyleneglycol and Acetylmethylcarbinol.*

By Arthur Harden, D.Sc., Ph.D., and George Stanley Walpole, B.Sc. (Melbourne).

(Communicated by Dr. C. J. Martin, F.R.S. Received December 5, 1905,—
Read February 1, 1906.)

(From the Chemical Laboratory, Lister Institute.)

## I. *Action of B. lactis aerogenes on Glucose.*

In the course of an investigation on the chemical action on glucose of the lactose-fermenting bacteria of fæces* it was found that, whereas *B. coli communis* yields, with glucose, acetic acid and alcohol in approximately equal molecular proportions, *B. lactis aerogenes* produces a much smaller amount of acetic acid, relatively both to the alcohol and to the sugar fermented. It was, moreover, previously known that *B. lactis aerogenes* produces a greater volume of gas, containing a larger proportion of carbon dioxide than does *B. coli communis.*† These facts indicate that the fermentation of glucose by this organism is of a different type from that brought about by the *B. coli communis.*‡ A detailed examination of the products was therefore made. For this purpose the organism was grown anaerobically in a medium containing 1 per cent. of Witte peptone and 2 per cent. of pure glucose in the presence of chalk, the method of collecting the gases evolved and of examining the products being the same as that previously employed in the investigation of the action of *B. coli communis* on glucose.§

All the substances produced by *B. coli communis* from glucose were again found, viz., lactic acid, acetic acid, succinic acid, formic acid, ethyl alcohol, carbon dioxide, and hydrogen. A quantitative estimation, however, revealed the fact that only about two-thirds of the carbon of the glucose was thus accounted for. This is shown in the following tables, which embody the results of three separate estimations (Cols. 1, 2, 3), Table I giving the actual percentages by weight on the sugar fermented of the various substances produced, and Table II the number of carbon atoms per molecule of sugar decomposed represented by each product. The results of a typical fermentation of glucose by *B. coli communis* are added (Col. 4) for the sake of comparison :—

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* Harden, 'Journ. of Hygiene,' 1905, vol. 5, p. 488.

† Theobald Smith, 'Centralbl. f. Bakteriol.,' 1895, vol. 18, pp. 1, 494, 589.

‡ Harden, 'Trans. Chem. Soc.,' 1901, p. 601.

§ Harden, *loc. cit.*

Table I.

| | 1. | 2. | 3. | 4. |
|---|---|---|---|---|
| Alcohol | 17 ·1 | 16 ·4 | 18 ·2 | 12 ·85 |
| Acetic acid | 5 ·1 | 4 ·2 | 8 ·6 | 18 ·84 |
| Lactic acid | 5 ·5 | 4 ·7 | 9 ·1 | 31 ·90 |
| Succinic acid | 2 ·4 | 3 ·1 | 4 ·5 | 5 ·20 |
| Formic acid | 1 ·0 | 0 ·75 | 1 ·7 | 0 ·0 |
| Carbon dioxide | 38 ·0 | — | 35 ·2 | 18 ·09 |
| Carbon dioxide, c.c. per gramme | 198 ·3 | — | 178 ·5 | 91 ·8 |
| Hydrogen, c.c. per gramme | 82 ·4 | — | 92 ·4 | 110 |
| Ratio $H_2/CO_2$ | 0 ·42 | — | 0 ·52 | 1 ·19 |
| Percentage excess of l-lactic acid | 50 ·0 | 66 ·0 | 86 ·0 | Not determined |

Table II.

| | 1. | 2. | 3. | 4. |
|---|---|---|---|---|
| Alcohol | 1 ·34 | 1 ·28 | 1 ·43 | 1 ·01 |
| Acetic acid | 0 ·31 | 0 ·25 | 0 ·52 | 1 ·13 |
| Lactic acid | 0 ·33 | 0 ·28 | 0 ·55 | 1 ·91 |
| Succinic acid | 0 ·15 | 0 ·19 | 0 ·27 | 0 ·32 |
| Formic acid | 0 ·04 | 0 ·02 | 0 ·07 | 0 ·00 |
| Carbon dioxide | 1 ·60 | — | 1 ·44 | 0 ·74 |
| Total | 3 ·77 | — | 4 ·28 | 5 ·11 |
| Hydrogen, atoms per molecule | 1 ·33 | — | 1 ·50 | 1 ·77 |

It will be observed that the ratio of hydrogen to carbon dioxide by volume is about 0·5 to 1, whilst these gases are produced by *B. coli communis* in approximately equal volumes. Theobald Smith,* using an ordinary fermentation tube, gives the characteristic ratio for *B. coli communis* as $H_2/CO_2 = 2:1$ and for *B. lactis aerogenes* $H_2/CO_2 = 1:1$. This difference is due to the solubility of the carbon dioxide in the liquid medium, and it must be remembered that while Smith's ratios give a perfectly satisfactory working test for the discrimination of the organisms, they do not represent the actual volumes or ratios of the gases produced.

Further examination of the fermentation products revealed the fact that no other acids had been formed, and search was therefore made for compounds of a different type. It was previously suggested that the deficiency of carbon observed in the fermentation produced by *B. coli communis*, amounting to only 0·25 to 0·9 of an atomic proportion of carbon, might possibly be due to the presence of reduction products of sugar, and compounds of this kind were therefore sought.

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* *Loc. cit.*

It was found that when the neutral liquid, containing the products of fermentation along with peptone, was evaporated to dryness at 55° under diminished pressure and extracted with alcohol, a solution was obtained which yielded on fractionation a colourless liquid boiling at 181° to 183° (corr.) at 760 mm. pressure. The yield was very small, only amounting to about 1 gramme per litre of medium containing 20 grammes of glucose, but it was found possible to increase the yield by employing a medium containing 5 per cent. of glucose, and in this way 8 grammes of the new substance, containing 52·8 per cent. of carbon, were obtained per litre of medium containing 50 grammes of sugar. This only accounts for about two-thirds of the missing carbon, and a rough estimate of the amount lost during the process of distillation and extraction was, therefore, made by dissolving 8 grammes of the material in 500 c.c. of a medium containing 5 grammes of Witte peptone, 6 grammes of calcium lactate and 6·5 grammes of alcohol and then extracting it in the manner described above. Only 5·2 grammes were recovered, the loss per 500 c.c. being therefore about 2·8 grammes and the loss per litre about 5·6 grammes. This brings the total amount produced from 50 grammes of glucose to about 13·6 grammes, slightly in excess of that required. It is hoped that the actual yield may be increased by a careful fractionation of the fermentation products.

The new product is apparently a mixture, and it has not yet been found possible to separate and identify all the components, so that the following must be taken as only a preliminary account of the substance.

It boils at 181° to 183° (corr.), and solidifies in the cold to a transparent mass which melts indefinitely at about 28°. It is optically active, the value for $[\alpha]_D$ for different preparations varying from 0·46 to 0·71. The composition of the substance dried by quicklime is approximately that of a butyleneglycol, but the percentage of carbon is about 0·6 too low. It does not reduce Fehling's solution either in the cold or on heating. That this substance contains a large proportion of 2 : 3-butyleneglycol, $CH_3.CH(OH).CH(OH).CH_3$, is shown by the following facts :—

1. When the liquid is heated with phenylisocyanate dissolved in anhydrous ether, combination occurs and a mixture of urethanes is produced. The fraction of these which is least soluble in alcohol comprises about 90 per cent.

of the whole amount and has the composition of the diphenylurethane of butyleneglycol ($C_4H_{10}O_2.2C_6H_5NCO$):—

Analysis.

| | Found. | Calculated. |
|---|---|---|
| C ............ | 65·79 | 65·85 |
| H ............ | 6·21 | 6·09 |
| N ............ | 8·57 | 8·53 |

It is sparingly soluble in cold alcohol, ether and benzene, crystallises in rosettes of needles and melts at 197° to 198° (uncorr.). When the urethane is boiled with baryta water or caustic soda solution it is decomposed and yields a glycol boiling at about the same temperature as the original material. This glycol has, however, not yet been isolated in the perfectly pure and dry state. A monourethane, $C_4H_{10}O_2C_6H_5NCO$, has also been prepared which is somewhat more soluble in cold alcohol than the diurethane, and crystallises in needles, melting at 100°:—

Analysis.

| | Found. | Calculated. |
|---|---|---|
| N ............... | 6·89 | 6·65 |

2. Both the crude glycol and that recovered from the diurethane are converted by oxidation with bromine in the light* into diacetyl, $CH_3.CO.CO.CH_3$ which was recognised by its extremely characteristic appearance and smell, and by the formation of a phenylosazone melting at 242° to 242°·8 (uncorr.).† The formation of this substance shows conclusively that 2 : 3-butyleneglycol must be present in the fermentation product.

*Detection of Acetylmethylcarbinol among the Fermentation Products.*—It was further found that the distillate from the liquid in which the organism was grown reduced Fehling's solution in the cold and gave with phenylhydrazine the osazone of diacetyl, melting at 243°. These properties point to the presence in the distillate of acetylmethylcarbinol, $CH_3.CO.CH(OH).CH_3$, which has previously been detected in this way by Grimbert‡ and by Desmots§ in the products of the fermentation of glucose by several bacteria: *B. tartricus, B. mesentericus vulgatus, B. fuscus, B. flavus, B. niger, B. ruber, B. subtilis,* and *Tyrothrix tenuis.* It has also been found in vinegar.

This compound appears only to be formed in very small amount. Since it is likewise converted into diacetyl by oxidation with bromine in the light, it is important to notice that the glycol used for conversion into diacetyl, as described above, was quite free from any substance capable of reducing Fehling's solution, and yielded a relatively large amount of diacetyl.

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* v. Pechmann, 'Ber.,' 1890, vol. 23, p. 2427.

† v. Pechmann, 'Ber.,' 1888, vol. 21, p. 2754.

‡ 'Compt. Rend.,' 1901, vol. 132, p. 706.

§ 'Compt. Rend.,' 1904, vol. 138, p. 581.

### II. *Action of B. lactis aerogenes on Mannitol.*

A quantitative examination of the products of fermentation of mannitol by *B. lactis aerogenes* showed that in this case also the action differed from that produced by *B. coli communis,* but that the deficit of carbon was only one-half of that found for glucose. This is shown in the following tables: Table III giving the percentages and Table IV the number of carbon atoms per molecule of mannitol represented by the products in two experiments (Cols. 1 and 2). As before, the products obtained by the action of *B. coli communis* are also given for the sake of comparison (Col. 3):—

Table III.

| | 1. | 2. | 3. |
|---|---|---|---|
| Alcohol | 32·5 | 32·5 | 28·1 |
| Acetic acid | 2·5 | 2·1 | 9·5 |
| Lactic acid | 8·6 | 8·6 | 18·6 |
| Succinic acid | 3·2 | 2·8 | 8·9 |
| Formic acid | 1·5 | 1·6 | 3·0 |
| Carbon dioxide | 35·5 | 35·5 | 28·44 |
| Carbon dioxide, c.c. per gramme | 180·3 | 180·3 | 143·0 |
| Hydrogen, c.c. per gramme | 138·3 | 143·6 | 167·0 |
| Ratio $H_2/CO_2$ | 0·77 | 0·79 | 1·18 |
| Percentage excess of l-lactic acid | 65·0 | 56·0 | 79·0 |

Table IV.

| | 1. | 2. | 3. |
|---|---|---|---|
| Alcohol | 2·57 | 2·57 | 2·22 |
| Acetic acid | 0·15 | 0·12 | 0·58 |
| Lactic acid | 0·52 | 0·52 | 1·13 |
| Succinic acid | 0·20 | 0·17 | 0·55 |
| Formic acid | 0·06 | 0·065 | 0·12 |
| Carbon dioxide | 1·47 | 1·47 | 1·16 |
| Total | 4·97 | 4·91 | 5·76 |
| H atoms per molecule glucose | 2·26 | 2·34 | 2·7 |

Further examination has shown that in this case as in that of glucose, both acetylmethylcarbinol and a glycol are produced, but both in much less quantity. The amount of crude glycol actually isolated from the products of fermentation of 50 grammes of mannitol was only 0·75 gramme. Since, however, the loss in isolating may be roughly taken as about 5 grammes, this is approximately the yield which would be expected if 6 to 7 grammes were formed. The nature of these products and their quantitative estimation, as well as the study of their optical properties, is still under investigation, and search is also being made for these and similar substances among the fermentation products of other bacteria.

### *General Considerations.*

The production of so large a proportion of 2:3-butyleneglycol in these experiments affords clear proof that this substance is derived from the glucose. The interesting question as to the mode of its production from the glucose or mannitol molecule will be best deferred until a more complete examination of the products, and especially of their optical relations, has been made. The close constitutional relation between the glycol and lactic acid, and the readiness with which its oxidation product—diacetyl—passes into an aromatic compound are also points of great interest. It may, however, be noted that the comparison of the fermentation products of *B. coli communis* and *B. lactis aerogenes* shows, firstly, that the alcohol produced by the latter organism is slightly greater in amount than that due to the former, and, secondly, that it is at the expense of that part of the molecule which in the *B. coli* fermentation yields acetic acid and lactic acid, that the *B. lactis aerogenes* forms the new products.

It may further be observed that both these bacteria produce twice as much alcohol from mannitol as from glucose, a fact which tends to confirm the suggestion previously made,* that the formation of alcohol in these reactions is related to the presence of the terminal $CH_2(OH).CH(OH)$ group, which occurs twice in the molecule of mannitol and only once in that of glucose.

A substance of the composition of butyleneglycol has previously been isolated from the products of fermentation of sugar by yeast,† and was also found in wine‡ and in brandy.§ This substance, boiled at 178° to 179°, yielded a diacetin boiling at 192° to 193°, and was considered to be identical with the synthetical isobutyleneglycol of Nevolé,[*] which boils at 176° to 178°. The yield obtained from sugar was, however, very small, and only amounted to about 0·2 per cent. after allowing for the losses involved in the extraction of the compound.

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* Harden, 'Trans. Chem. Soc.,' 1901, p. 601.

† Claudon and Morin, 'Compt. Rend.,' 1887, vol. 104, p. 1109; Henninger and Sanson, 'Compt. Rend.,' 1888, vol. 106, p. 208.

‡ Henninger, 'Compt. Rend.,' 1882, vol. 95, p. 94.

§ Morin, 'Compt. Rend.,' 1887, vol. 105, p. 1019.

In view of the properties of the crude glycol described above, it would seem advisable to re-examine Henninger's glycol, the constitution of which was not experimentally examined.

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# *The Alcoholic Ferment of Yeast-Juice.*

By Arthur Harden, D.Sc., Ph.D., and William John Young, M.Sc.

(Communicated by Dr. C. J. Martin, F.R.S. Received December 8, 1905,—Read February 1, 1906.)

(From the Chemical Laboratory, Lister Institute.)

## 1. *Effect of the addition of Boiled and Filtered Yeast-juice on the Fermentation of Glucose Produced by Yeast-juice.*

In the course of some experiments on the action of various proteids on the fermentative activity of yeast-juice, it was observed that the alcoholic fermentation of glucose by yeast-juice is greatly increased by the addition of yeast-juice which has been boiled and filtered, either when fresh or after having undergone autolysis, although this boiled liquid is itself incapable of setting up fermentation. Thus, the total fermentation produced by yeast-juice acting on excess of glucose is, as a rule, doubled by the addition of an equal volume of the boiled juice, and a further increase is produced when a greater volume is added, the sugar concentration being kept constant.[†]

A similar observation was previously made by Buchner and Rapp[‡] in a single experiment (No. 265).

The following table embodies a few of the results obtained, the yeast-juice being prepared and the amount of carbon dioxide evolved being estimated by the method previously employed by the authors.* In every case the concentration of sugar was kept constant, and both in these and all the fermentation experiments described in this paper, toluene was added as an antiseptic.

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[*] 'Compt. Rend.,' 1876, vol. 83, p. 65.

[†] Harden and Young, Preliminary Note, 'Proc. Physiol. Soc.,' 1904, vol. 32, November 12.

[‡] 'Ber.,' 1899, vol. 32, p. 2093.

Table I.—Effect of the Addition of Boiled Yeast-juice on the Total Fermentation of Glucose by Yeast-juice.

| No. | Juice. | Water. | Boiled juice. | Glucose. | Time. | Carbon dioxide. |
|---|---|---|---|---|---|---|
| | c.c. | c.c. | c.c. | grammes. | hours. | gramme. |
| 1 ...... | 25 | 25 | 0 | 5 | 72 | 0·137 |
| | 25 | 0 | 25 | 5 | 72 | 0·378 |
| 2 ...... | 20 | 20 | 0 | 4 | 44 | 0·115 |
| | 20 | 0 | 20 | 4 | 44 | 0·363 |
| 3 ...... | 25 | 0 | 0 | 2·5 | 40 | 0·370 |
| | 25 | 0 | 25 | 5 | 40 | 0·620 |
| 4 ...... | 20 | 40 | 0 | 6 | 42 | 0·458 |
| | 20 | 0 | 40 | 6 | 42 | 0·858 |
| 5 ...... | 25 | 25 | 0 | 5 | 44 | 0·346 |
| | 25 | 0 | 25 | 5 | 44 | 0·709 |
| 6 ...... | 25 | 25 | 0 | 5 | 48 | 0·110 |
| | 25 | 0 | 25 | 5 | 48 | 0·216 |
| 7 ...... | 25 | 25 | 0 | 5 | 60 | 0·273 |
| | 25 | 0 | 25 | 5 | 60 | 0·466 |
| 8 ...... | 25 | 25 | 0 | 5 | 120 | 0·424 |
| | 25 | 0 | 25 | 5 | 120 | 0·959 |
| 9 ...... | 25 | 25 | 0 | 5 | 72 | 0·414 |
| | 25 | 20 | 5 | 5 | 72 | 0·546 |
| | 25 | 10 | 15 | 5 | 72 | 0·735 |
| | 25 | 5 | 20 | 5 | 72 | 0·810 |
| | 25 | 0 | 25 | 5 | 72 | 0·924 |
| 10 ...... | 25 | 25 | 0 | 5 | 70 | 0·246 |
| | 25 | 0 | 25 | 5 | 70 | 0·356 |
| | 25 | 50 | 0 | 7·5 | 70 | 0·180 |
| | 25 | 0 | 50 | 7·5 | 70 | 0·431 |
| | 25 | 75 | 0 | 10 | 70 | 0·141 |
| | 25 | 0 | 75 | 10 | 70 | 0·515 |

In Experiments 1 to 5 the juice added had been autolysed before being boiled; in Nos. 6 to 8 the added juice was boiled as soon as it had been prepared. Experiments 9 and 10 show that each successive addition of boiled juice, from 0·2 to 3 volumes, produces a further increase in the amount of the fermentation.

A similar effect is produced, (1) By the precipitate produced in boiled yeast-juice by the addition of 3 volumes of alcohol (Experiment 1, Table II); (2) By the liquid formed by the autoplasmolysis of yeast, when it is allowed to stand at the air temperature for some time (Experiments 2 and 3, Table II); (3) By the liquid obtained by boiling Buchner's "Aceton-Dauerhefe" with water (Experiment 4, Table II). Further, yeast killed by acetone and ether (Aceton-Dauerhefe) reacts with boiled juice in the same way as does yeast-juice (Experiment 5, Table II).

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* Harden and Young, 'Ber.,' 1904, vol. 37, p. 1052.

Table II.—Effect of Various Substances in Increasing Alcoholic Fermentation.

| No. | Yeast-juice. | Addition. | Glucose. | Time. | Carbon dioxide. |
|---|---|---|---|---|---|
| | c.c. | | | hours. | gramme. |
| 1 | 25 | 25 c.c. water | 5 | 48 | 0·110 |
| | 25 | 25 c.c. water + precipitate by 75 per cent. alcohol from 25 c.c. boiled fresh juice | 5 | 48 | 0·268 |
| | 25 | Filtrate from 25 c.c. boiled fresh juice + 3 volumes alcohol, made to 25 c.c. | 5 | 48 | 0·141 |
| | 25 | 25 c.c. water + precipitate from 25 c.c. boiled old juice by 75 per cent. alcohol | 5 | 48 | 0·286 |
| 2 | 25 | 25 c.c. water | 5 | 72 | 0·070 |
| | 25 | 25 c.c. autoplasmolysed yeast-juice, made neutral | 5 | 72 | 0·189 |
| 3 | 25 | 25 c.c. water | 5 | 72 | 0·084 |
| | 25 | 25 c.c. autoplasmolysed yeast-juice, made neutral | 5 | 72 | 0·172 |
| | 25 | 25 c.c. water | 5 | 72 | 0·475 |
| 4 | 25 | 25 c.c. aqueous infusion of 2 grammes Aceton-Dauerhefe | 5 | 72 | 0·625 |
| 5 | 2 grammes Aceton-Dauerhefe | 40 c.c. water | 4 | 48 | 0·062 |
| | ” | 20 c.c. water + 20 c.c. boiled juice | 4 | 48 | 0·136 |

## 2. *Dialysis of the Boiled Juice.*

The constituent of the boiled and filtered juice to which this effect is due is removed when the liquid is dialysed in a parchment tube, leaving an inactive residue. In the experiments detailed in the following table (Table III, Experiments 1, 2 and 3) the effect of the addition of boiled juice is compared with that produced by the residue and dialysate respectively.

In Experiment 4, the unboiled juice was dialysed, and the fact that the dialysate had a similar effect to a boiled juice shows that the active constituent exists in the original yeast-juice and is not formed during the boiling.

Table III.—Dialysis of Boiled Yeast-juice. 25 c.c. yeast-juice + 5 grammes glucose + toluene.

| No. | Water. | Boiled juice. | Residue. | Dialysate. | Time. | Carbon dioxide. |
|---|---|---|---|---|---|---|
| | c.c. | c.c. | c.c. | c.c. | hours. | gramme. |
| 1 | 25 | 0 | 0 | 0 | 48 | 0·253 |
| | 0 | 25 | 0 | 0 | 48 | 0·561 |
| | 0 | 0 | 25 | 0 | 48 | 0·264 |
| 2 | 25 | 0 | 0 | 0 | 48 | 0·268 |
| | 0 | 25 | 0 | 0 | 48 | 0·497 |
| | 0 | 0 | 25 | 0 | 48 | 0·276 |
| 3 | 25 | 0 | 0 | 0 | 72 | 0·113 |
| | 0 | 25 | 0 | 0 | 72 | 0·334 |
| | 0 | 0 | 25 | 0 | 72 | 0·189 |
| | 0 | 0 | 0 | 25 | 72 | 0·334 |
| 4 | 25 | 0 | 0 | 0 | 48 | 0·154 |
| | 0 | 0 | 0 | 25 | 48 | 0·251 |

### 3. *Dialysis of Yeast-juice.*

The facts above detailed suggested the possibility of dividing yeast-juice into two fractions by dialysis; an inactive residue and a dialysate which, although itself inert, would be capable of rendering this residue active.

This was experimentally realised by filtering the juice through a Martin gelatin filter.*

This method of rapid dialysis was chosen because the yeast-juices at our disposal lost their activity too rapidly to permit of the ordinary process of dialysis through parchment being carried out. Either a 10- or a 7·5-per-cent. solution of gelatin was used to impregnate the Chamberland filter and the filtration was carried out under a pressure of 50 atmospheres.

Only a portion of the juice placed in the filter was actually filtered, the remainder being simply poured out of the case as soon as a sufficient quantity of filtrate had passed through. The residue adhering to the candle, which consisted of a brown viscid mass, was dissolved in water and made up to the volume of the juice filtered. Glucose was then added and one portion incubated at 25° with an equal volume of sugar solution and a second portion with an equal volume of the filtrate or of a boiled juice, containing an equal amount of glucose. Before incubation the carbon dioxide was pumped out of all the solutions. The filtrate was invariably found to be quite devoid of fermenting power, none of the enzyme having passed through the gelatin.

* 'Journ. Physiol.,' 1896, vol. 20, p. 364.

The results (Table IV) show that in this way an almost inactive residue can be obtained which is rendered active by the addition of the filtrate (Experiments 1, 2, 3) or a boiled juice (Experiment 4).

Table IV.—Filtration of Yeast-juice through the Martin Gelatin Filter.
15 c.c. residue + 3 grammes glucose + toluene.

| No. | Water. | Filtrate. | Boiled juice. | Time. | Carbon dioxide. |
|---|---|---|---|---|---|
| | c.c. | c.c. | c.c. | hours. | gramme. |
| 1 | 15 | 0 | 0 | 48 | 0·000 |
| | 0 | 15 | 0 | 48 | 0·035 |
| 2 | 15 | 0 | 0 | 60 | 0·001 |
| | 0 | 15 | 0 | 60 | 0·051 |
| 3 | 15 | 0 | 0 | 60 | 0·008 |
| | 0 | 15 | 0 | 60 | 0·064 |
| 4 | 15 | 0 | 0 | 60 | 0·024 |
| | 0 | 0 | 15 | 60 | 0·282 |

The total fermentations observed even in the presence of the filtrate are very low, this being, at all events in part, due to the fact that in this series of experiments the original juices themselves happened to be of low fermenting power.

In a second set of experiments (Table V) a smaller quantity of juice was placed in the filter and the filtration was continued until no more liquid would pass through. The residue was then washed several times by adding water and forcing it through the filter. The time occupied in this process varied greatly with different juices, the limits for the filtration and washing of 50 c.c. of juice, using two filters simultaneously, were about 6 to 12 hours. The carbon dioxide was not estimated by absorption in potash as in the previous cases, but was collected and measured over mercury, by means of the apparatus described later on, the object of this procedure being to ascertain not only the total amount of carbon dioxide produced, but the rate and duration of the evolution. The residue was dissolved in water and made to the same volume as the original juice, and the filtrate was evaporated down to the same volume. All the solutions were saturated with carbon dioxide at the temperature of the bath (25°) before the measurements were commenced, and the observations were continued until all fermentation had ceased.

The boiled juice added in Experiments 1, 3 and 4 (Table V) was obtained by boiling a portion of the same preparation as was used for the filtration. The carbon dioxide is expressed in cubic centimetres under atmospheric conditions.

Table V.—Filtration of Yeast-juice through the Martin Gelatin Filter.

| No. | Vol. of juice filtered. | Wash water. | Residue. | Filtrate. | Boiled juice. | Glucose. | Carbon dioxide. |
|---|---|---|---|---|---|---|---|
|  | c.c. | c.c. | c.c. | c.c. | c.c. | grammes. | c.c. |
| 1 | 75 | 200 | 25 | 0 | 0 | 2·5 | 10·4 |
|  |  |  | 25 | 0 | 25 | 5 | 396·3 |
| 2 | 80 | 260 | 20 | 0 | 0 | 2 | 8·3 |
|  |  |  | 20 | 20 | 0 | 4 | 90·2 |
| 3 | 100 | 250 | 25 | 0 | 0 | 2·5 | 0·4 |
|  |  |  | 25 | 0 | 25 | 5 | 268 |
| 4 | 50 | 200 | 25 | 0 | 0 | 2·5 | 0·9 |
|  |  |  | 25 | 0 | 25 | 5 | 192 |

The process of filtration does not always produce an inactive residue, as on several occasions the residue after very thorough washing has been found to retain a considerable amount of activity. No reason has yet been found for this and it has not yet been ascertained whether it is due to some peculiarity in the particular specimen of juice or in the special filter employed.

It is of interest to note that in Experiment 2 (Table V) the residue alone gave 8·3 c.c. of carbon dioxide in 3 hours, the amount evolved in the last hour being only 0·1 c.c. At the close of this period the liquid still contained the alcoholic enzyme, since on the addition of 20 c.c. of the filtrate, fermentation recommenced and continued for many hours.

*These two sets of experiments (Tables IV and V) show that the fermentation of glucose by yeast-juice is dependent upon the presence of a dialysable substance which is not destroyed by heat.*

### 4. *Analysis of the Effect of the Addition of Boiled Juice upon the Fermentation of Glucose by Yeast-juice.*

In order to compare the course of the fermentation in the presence and in the absence of boiled yeast-juice, experiments were carried out in which the rate of evolution of carbon dioxide was observed in each case throughout the whole period of activity of the juice, which, as a rule, in presence of an excess of sugar, lasts for about 48 to 60 hours.

For this purpose the fermentation was allowed to proceed in a 100 c.c. flask, kept at the constant temperature of 25° by immersion in a thermostat, and connected with an azotometer, in which the gas was collected over mercury. The gas in the fermentation flask was maintained at a constant pressure, as nearly as possible that of the atmosphere, by keeping the mercury in the reservoir at a fixed level, by means of a syphon dipping into a small beaker. The volume of the gas was read on the azotometer without disturbing the mercury reservoir and was reduced to atmospheric pressure by means of a calibration curve. Since yeast-juice readily becomes supersaturated with carbon dioxide, the contents of the flask were vigorously shaken before each reading of the volume of gas. Before the observations were commenced the liquids were brought to the temperature of the thermostat, and were saturated with carbon dioxide. In all comparative experiments the concentration of glucose was the same.

When the rates of evolution of carbon dioxide from (1) a solution of glucose in yeast-juice, and (2) a similar solution to which boiled and filtered yeast-juice has been added are compared, it is found that two phenomena are concerned in the production of the increased fermentation in the presence of boiled yeast-juice.

(*a*) An initial rapid evolution of carbon dioxide is produced, which soon diminishes until a rate is attained which remains nearly constant for several hours and is usually, but not invariably, approximately equal to that given by an equal volume of the same yeast-juice and glucose to which no addition has been made.

(*b*) The fermentation rate diminishes more slowly, so that the fermentation continues for a longer period. The greater proportion of the total increase is usually due to this second phenomenon.

The results obtained in a typical experiment of this kind are shown in Fig. 1. The initial period of the evolution is plotted separately (Curves A′ and B′) on a larger scale.

Curves A and A′ in which the evolution of carbon dioxide is plotted against time represent the course of a fermentation with 25 c.c. yeast-juice + 25 c.c. water + 5 grammes glucose + toluene. The rate to begin with is 48 c.c. per hour, but rapidly decreases until it becomes equal to 24 c.c. per hour, at which it remains almost constant for about 5 hours, gradually decreasing until, after the expiration of about 40 to 45 hours, fermentation ceases. The total evolution amounted to 369 c.c. under atmospheric conditions.

Curves B and B′ refer to 25 c.c. of the same yeast-juice + 25 c.c. of a boiled yeast-juice + 5 grammes glucose + toluene. The initial rate is much higher, 168 c.c. per hour, but this falls gradually in the course of 40 minutes to 30 c.c. per hour. This rate of 30 c.c. per hour falls off much less rapidly than that in Experiment A, the fermentation continuing for about 80 to 85 hours and yielding in all 1174 c.c. of carbon dioxide. It is important to bear in mind that these curves represent the gradual disappearance of the fermenting power

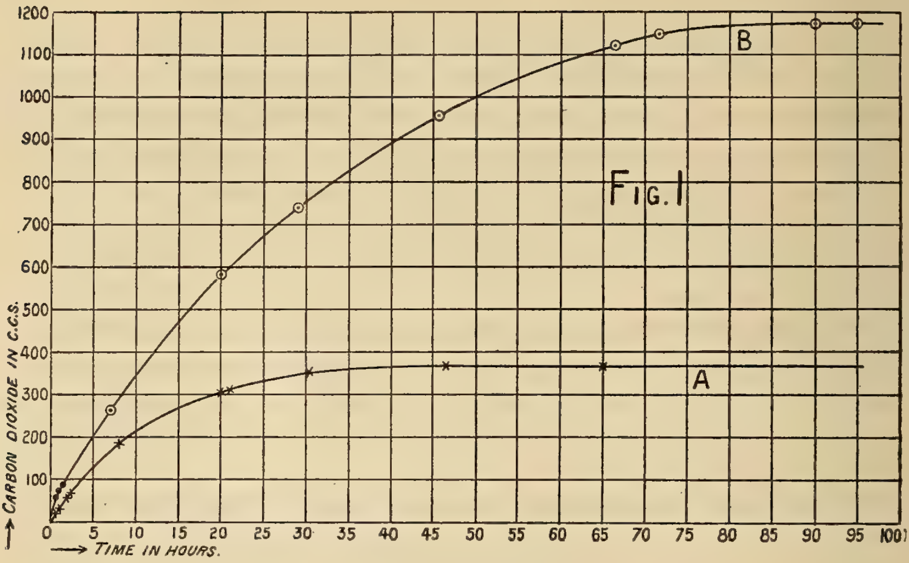
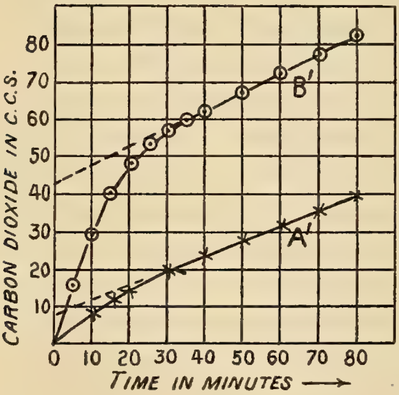


FIG. 1



of the liquid, and not the diminution of the amount of fermentation with diminishing concentration of sugar, an excess of this substance being present throughout.

A comparison of the two curves shows very clearly the two factors involved in the great increase in Experiment B: (1) The initial rapid evolution, and (2) the prolongation of the fermentation.

### 5. *The Initial Period of Rapid Evolution of Carbon Dioxide.*

This is a very striking phenomenon, and a typical example is illustrated in fig. 2 in which the curves show the course of the evolution of carbon dioxide (total volume evolved plotted against time) during two hours in the case of:

A. 25 c.c. yeast-juice + 75 c.c. water + 10 grammes glucose + toluene.

B. 25 c.c. yeast-juice + 50 c.c. water + 25 c.c. boiled autolysed yeast-juice + 10 grammes glucose + toluene.

C. 25 c.c. yeast-juice + 75 c.c. boiled autolysed yeast-juice + 10 grammes glucose + toluene.

In B and C the initial rates are almost equal (58 c.c. in 10 minutes) and much greater than in A (14 c.c. in 10 minutes). In B the rate rapidly falls off whilst in C it diminishes much more slowly. A similar initial period is also observable in A, but is not nearly so marked.

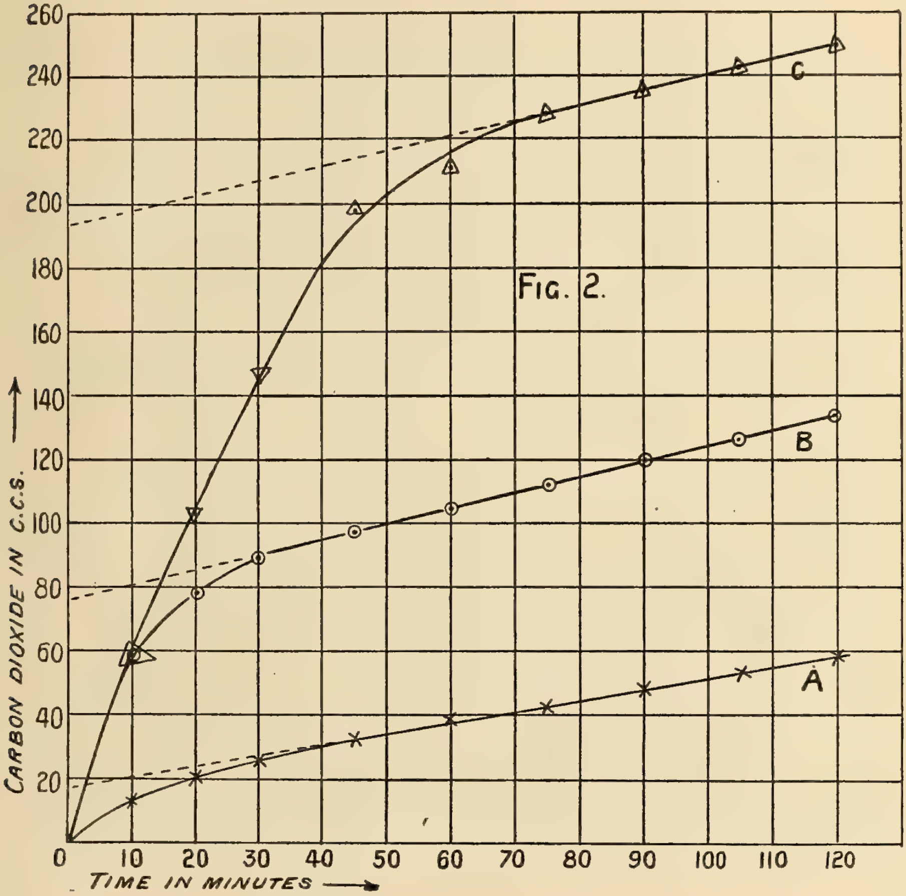


Fig. 2.

The extra quantity of carbon dioxide evolved in this initial period may be calculated by subtracting the amount corresponding with the constant rate which is finally attained from the total amount observed. This is done graphically in fig. 2 by continuing the straight line representing the constant rate back to the axis of ordinates. The following numbers are thus obtained: for A, 16·6; for B, 75·4; for C, 192·9.

The amounts due to the addition of boiled juice are therefore: for 1 volume in B, 75·4 − 16·6 = 58·8; for 3 volumes in C, 192·9 − 16·6 = 176·3 = 3 × 58·8.

*The extra amount of carbon dioxide is, therefore, directly proportional to the volume of boiled juice added.*

### 6. *Production of the Initial Rapid Evolution of Carbon Dioxide by the Addition of Phosphates.*

As the result of a large number of attempts to isolate the constituent of boiled juice which brings about the increase in fermentation, it was found that whenever an increase was produced phosphoric acid in the form of a soluble phosphate was present. The effect of the addition of soluble phosphates to yeast-juice was, therefore, examined and it was found that a well-marked initial rapid evolution of carbon dioxide was thus produced. Since, moreover, the boiled juices employed invariably contained phosphates, precipitable by magnesia mixture, there can be no doubt that it is to the presence of these that this initial phenomenon is due. Quantitative estimations revealed the somewhat surprising fact that the extra quantity of carbon dioxide evolved in the initial period when a phosphate or a boiled juice is added, corresponds with the evolution of one molecular proportion of carbon dioxide for each atom of phosphorus added in the form of phosphate.

In order to obtain accurate results with solutions of sodium or potassium phosphate, the fact that these absorb carbon dioxide must be taken into consideration. Solutions of the dihydrogen salts of potassium and sodium are too acid to be employed and the monohydrogen salts or a mixture of these with the dihydrogen salts were always used. In every case the liquid before being added to the yeast-juice was saturated with carbon dioxide at the temperature of the bath, and the volume of carbon dioxide liberated by the addition of excess of hydrochloric acid was ascertained in an aliquot portion.

At the close of the fermentation the fermented liquid was acidified and the residual combined carbon dioxide measured, the difference between this and the original amount being subtracted from the amount evolved during the fermentation.

The results are more precise when the yeast-juice employed is an active one, since when the fermenting power of the juice is low the initial period becomes unduly prolonged and the calculation of the extra amount of carbon dioxide is rendered uncertain. The equivalence of the carbon dioxide and phosphate is established by the results contained in the following Table VI. Column 1 gives the observed amount of extra carbon dioxide calculated as described above and reduced to grammes, and Column 2 the equivalent of the phosphate added, this being estimated by precipitation with magnesium citrate mixture in the boiled juice or phosphate solution.

In Experiments 1 to 7 boiled juice was added; in 8 to 14 a solution of sodium or potassium phosphate.

The maximum rate attained during the initial period is from five to eight times as high as the constant rate attained after the evolution of the carbon dioxide equivalent to the phosphate present.

Table VI.—Equivalence of Extra Carbon Dioxide Evolved during the Initial Period, and Phosphate added.

| Experiments. | Grammes of carbon dioxide. | | Experiments. | Grammes of carbon dioxide. | |
|---|---|---|---|---|---|
| | Column I—Observed. | Column II—Calculated from phosphate. | | Column I—Observed. | Column II—Calculated from phosphate. |
| 1 | 0·090 | 0·086 | 8 | 0·196 | 0·197 |
| 2 | 0·054 | 0·055 | 9 | 0·066 | 0·065 |
| 3 | 0·058 | 0·051 | 10 | 0·057 | 0·061 |
| 4 | 0·060 | 0·049 | 11 | 0·056 | 0·061 |
| 5 | 0·106 | 0·112 | 12 | 0·059 | 0·061 |
| 6 | 0·103 | 0·101 | 13 | 0·068 | 0·070 |
| 7 | 0·113 | 0·112 | 14 | 0·071 | 0·070 |

At the commencement of the period when sodium or potassium phosphate solution has been added, the rate only gradually acquires its maximum value and sometimes it only attains this maximum after a considerable interval.

This phenomenon is occasionally observed in the fermentation produced by yeast-juice without the addition of phosphate, and also sometimes occurs, but to a much smaller extent, when boiled juice is added. It is well shown in Curve B, fig. 3, which represents the fermentation produced by 25 c.c. yeast-juice + 25 c.c. of a 0·06 molar solution of sodium phosphate + 5 grammes glucose + toluene. The cause of this period of induction has not yet been ascertained.

### 7. *Limit of the Action of Phosphate.*

If the fermentation in presence of phosphate be allowed to continue until the steady rate is attained and a second quantity of phosphate be then added, a second period of rapid evolution of carbon dioxide sets in and proceeds in a similar manner to the first. This is shown in Curves B and C, fig. 3, which represent the effect of the successive addition of two quantities of 5 c.c. of 0·3 molar sodium phosphate to 25 c.c. yeast-juice + 20 c.c. water, in presence of 10 per cent. glucose. Curve A represents the fermentation in absence of added phosphate. The phosphate solution employed was a mixture of five molecules of $NaH_2PO_4$ with one molecule of $Na_2HPO_4$ and no correction for combined carbon dioxide was required. The extra amount of carbon dioxide evolved after each addition is the same, and is equivalent, as already stated, to the phosphate added. The equality is shown graphically in the curve and the equivalence in Experiments 13 and 14, Table VI.

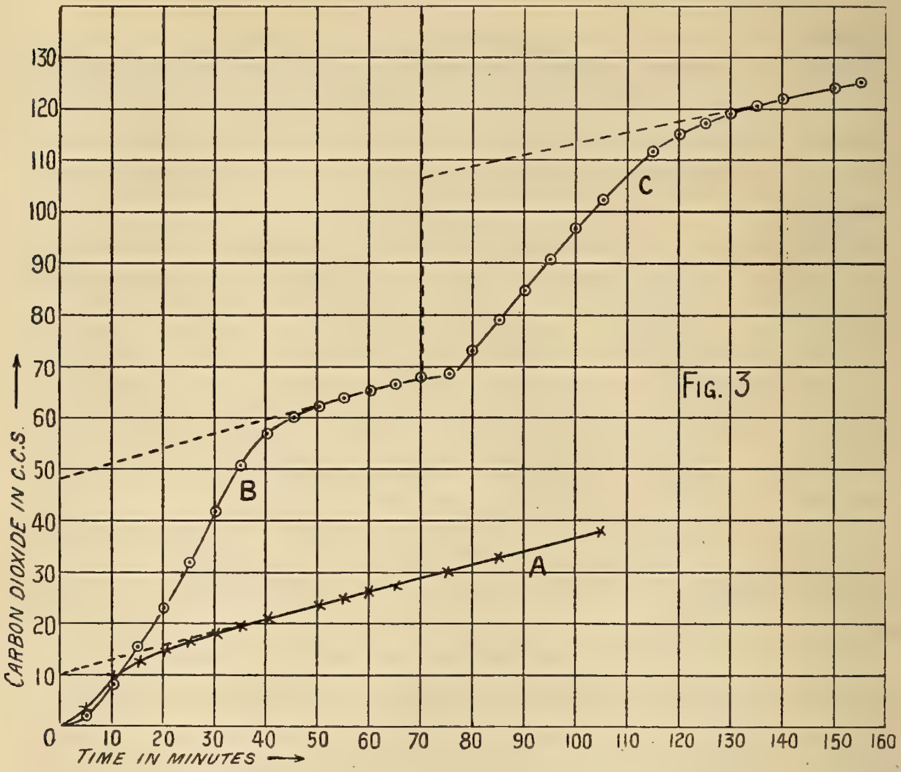


FIG. 3

This process cannot, however, be repeated indefinitely, as after a certain limit is reached the reaction no longer occurs and with a large excess the fermentation is stopped. The exact limit appears to vary both with the nature of the phosphate added and with the particular specimen of yeast-juice employed. The greatest amount of carbon dioxide hitherto obtained in this way from 25 c.c. of yeast-juice is about 0·45 gramme (230 c.c.), which was observed on two occasions, once after the addition of four volumes of boiled juice, and again after the addition of 50 c.c. of a solution of a mixed magnesium potassium phosphate yielding with magnesia mixture 1·187 grammes of magnesium pyrophosphate.

When a specimen of yeast-juice has been incubated until it will no longer ferment sugar, it is not affected by the addition of phosphate.

The fact that the extra carbon dioxide calculated in this way is equivalent to the phosphate present, suggests the superposition of two actions. Whether this is to be explained by the presence of two distinct enzymes or simply by the increased activity of a single enzyme remains to be decided.

### 8. *Products of Fermentation in the Presence of Phosphate.*

The carbon dioxide evolved during the initial period after the addition of a phosphate is the product of a true alcoholic fermentation of the glucose, in which alcohol and carbon dioxide are produced in equivalent amounts. This was proved in the following way. Twenty-five cubic centimetres of a solution containing 2·5 grammes of glucose and 5 c.c. of a 0·3 molar solution of potassium phosphate were added to 25 c.c. of yeast-juice; the mixture was incubated and the carbon dioxide collected and measured.

As soon as the rate of evolution had become constant, a further addition of 10 c.c. of 0·3 molar phosphate solution was made and the fermentation again continued until the rate had become constant. The gas evolved was tested and found to be carbon dioxide. The total amount evolved during the experiment, which lasted for 2 hours 10 minutes, was 163·4 c.c. at 19°·6 and 758·6 mm. or 0·291 gramme, the equivalent of the phosphate added being 0·196 gramme. The liquid was then distilled with steam and the alcohol estimated in the distillate, 1·312 grammes being found to be present. Twenty-five cubic centimetres of the original juice were found to contain 0·983 gramme of alcohol and therefore $1·312-0·983 = 0·329$ gramme were formed by the fermentation of the sugar. The ratio of alcohol to carbon dioxide produced is therefore $0·329/0·291 = 1·13$, which agrees well with the ratio previously found by similar methods for the fermentation of glucose by yeast-juice.* The theoretical ratio is 1·04.

Lactic acid and acetic acid were also estimated in the original juice and after fermentation in presence of phosphate, but only a very small variation was observed. Twenty-five cubic centimetres of juice gave before fermentation 0·122 gramme of zinc lactate and 0·083 gramme of acetic acid, and after fermentation 0·102 gramme of zinc lactate and 0·072 gramme of acetic acid.

### 9. *Fate of the Phosphoric Acid.*

When the fermented liquid is boiled and filtered almost the whole of the phosphorus present is found in the filtrate, but it is nearly all in a form which is not precipitated by ammoniacal magnesium citrate mixture.

In the following experiment three quantities of 25 c.c. of yeast-juice were taken:—

A. Hot water was added, the solution heated in a boiling water-bath and the coagulate filtered off and well washed.

B. Ten cubic centimetres of a 30 per cent. glucose solution and 10 c.c. of

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* Harden and Young, 'Ber.,' 1904, vol. 37, p. 1052.

0·3 molar potassium phosphate solution were added and the liquid at once heated to the boiling point, filtered, and the coagulate washed.

C. The same additions were made as to B and the liquid then fermented until the close of the initial period, after which it was heated and filtered like the others.

The total phosphorus was then estimated in each of the coagulates and in each of the filtrates, and the phosphorus precipitated by magnesium citrate in each of the three filtrates. The estimations of total phosphorus were made by heating with sulphuric and nitric acids until colourless, diluting and precipitating with magnesium citrate mixture in presence of excess of ammonia.

The following were the results obtained, the numbers representing the grammes of magnesium pyrophosphate per 25 c.c. of juice.

Table VII.

| | A. Original juice. | B. Juice + phosphate. Not fermented. | C. Juice + phosphate. Fermented. |
|---|---|---|---|
| Coagulate ............... | 0·053 | 0·057 | 0·072 |
| Filtrate— | | | |
| (*a*) Precipitated by Mg citrate ......... | 0·126 | 0·480 | 0·070 |
| (*b*) Not precipitated by Mg citrate ... | 0·271 | 0·282 | 0·679 |
| Total ...... | 0·450 | 0·819 | 0·821 |

The amount of phosphate added was equivalent to 0·372 gramme of magnesium pyrophosphate.

A number of other results are given to show the extent to which phosphate is converted into the non-precipitable form by this reaction. All the estimations were made by boiling and filtering the fermented liquid immediately upon the close of the initial period. As before the numbers represent grammes of magnesium pyrophosphate obtained from 25 c.c. of juice.

The form in which this non-precipitable phosphorus is actually present in the fermented liquid, and in the liquid which has been boiled and filtered, has not yet been ascertained with certainty. Experiments which are still in progress, however, appear to indicate that it exists in combination with glucose, probably in the form of a phosphoric ester.

Table VIII.—Conversion of Phosphate into the Non-precipitable Form by Yeast-juice and Glucose.

| | Phosphate added. | Precipitable phosphate in filtrate. | Non-precipitable phosphate in filtrate. |
|---|---|---|---|
| 1 | 0·553 | 0·066 | 1·032 |
| 2 | 0·490 | 0·090 | 0·832 |
| 3 | 0·250 | 0·054 | 0·685 |
| 4 | 0·488 | 0·091 | 1·040 |
| 5 | 0·495 | 0·088 | 0·881 |

The question as to whether the entire phenomenon of the fermentation of glucose by yeast-juice depends on the presence of phosphates has not yet been definitely decided. The addition of phosphate undoubtedly produces a larger increase in the total fermentation than is simply due to the equivalent amount of carbon dioxide evolved in the initial period. The extent of this increase appears to vary very considerably with different specimens of yeast-juice, but the prolongation of the fermentation is not so great as is caused by boiled fresh juice. This question can only be satisfactorily settled by ascertaining whether the addition of a phosphate to the perfectly inactive residue obtained from a juice by filtration through a gelatin filter is sufficient to restore its fermenting power in the same way as the filtrate or a boiled juice. Experiments on this point are in progress, but no decisive result has as yet been obtained, and all discussion of this point will best be deferred until these are completed.

Various other points of interest raised in the course of the investigation, and the study of the relation of these phenomena to the fermentation of glucose by living yeast, are also occupying our attention.

A short outline of the main conclusions arrived at in the foregoing paper, has been previously published in the form of two preliminary communications, without any experimental details.* After the appearance of these notes, Buchner and Antoni† repeated and confirmed a number of the experiments dealing with the effect of boiled juice and of phosphates on the total fermentation, and with the separation of the juice by dialysis into an inactive residue and a dialysate capable of rendering it active. Buchner and Antoni were able, with the more stable juice at their disposal, to carry out the dialysis in the ordinary way for 24 hours and in this manner to confirm the results obtained by the use of the gelatin filter. Owing to the lack of experimental detail, Buchner and Antoni imagined that in our comparative experiments the concentration of glucose and of enzyme had not been kept constant, and ascribed part of the increase produced by boiled juice to the favourable effect of a diminution in the concentration of the sugar and of the alcohol, which is always present, by dilution with the added boiled juice. The details given above show that neither of these influences had any share in the effects observed by us.

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* 'Journ. Physiol.,' 1904, vol. 32; 'Proc.,' of November 12; 'Proc. Chem. Soc.,' 1905, vol. 21, p. 189, June 6.

† 'Zeit. Physiol. Chem.,' 1905, vol. 46, p. 136.

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## *The Quantitative Estimation of Small Quantities of Nickel in Organic Substances.*

By H. W. Armit and A. Harden, D.Sc., Ph.D.

(Communicated by Dr. C. J. Martin, F.R.S. Received December 5, 1905,—Read February 1, 1906.)

(From the Chemical Department of the Lister Institute of Preventive Medicine.)

In the course of an investigation into the toxic action of certain nickel compounds, it was found necessary to devise a method of detecting and estimating nickel, when included in animal tissue, in quantities not exceeding a few milligrammes per cent.

A method has therefore been worked out, which, although in many respects only differing from the usual methods in virtue of slight alterations of detail, is capable of demonstrating extremely small quantities of nickel accurately. The method may be divided into three stages: (1) The Ashing; (2) The Separating; and (3) The Estimating stages.

1. *Ashing.*—The substance to be examined must be placed in a porcelain crucible (platinum is unsuitable, as a considerable loss of nickel takes place, probably by an alloy of platinum and nickel being formed) and evaporated to dryness over a water bath. If the substance be solid, it should be cut up into small pieces. The crucible is then heated carefully with a Bunsen flame, but it may be wise to further dry in a hot air oven or on a sand bath before this. Then it is burned over a Fletcher burner, and lastly fully incinerated in the blow-pipe flame. With some care, it is possible, as a rule, to oxidise fully all the carbon, without recourse to any foreign material. The crucible is then placed on the water bath, and 10 c.c. of pure hydrochloric acid are added and allowed to evaporate to dryness, this process being repeated. The residue is then extracted with water to which a small quantity of hydro-

chloric acid is added. For this purpose, 2 c.c. of a four times normal acid are generally employed. The extract is then filtered. The ash so obtained is practically completely soluble.

Ashing by Kjeldahl's method, or better, with sulphuric and nitric acids, can also be employed, but has two disadvantages over the simple incineration method: Firstly, it takes longer; and secondly, it introduces foreign salts, which should, if possible, be avoided.

2. *Separation.*—Firstly, it is necessary to get rid of the iron and at the same time of the phosphates. Those tissues, which contain iron in excess, *e.g.*, blood, may be treated by precipitation with excess of ammonia and filtration. The process should be repeated three times, the precipitate being redissolved each time with the same quantity of acid as was used for the extraction. If some of the iron separates out from the filtrate on being evaporated, it may be necessary to refilter before dryness is reached. When the substance contains little or no iron, it is necessary to add a sufficiency to combine with the quantity of phosphates present. If the phosphates are present in excess, the following method is employed. The cold solution is made neutral to litmus or very faintly acid with ammonia. An excess of ammonium acetate is then added (as a rule 8 to 10 c.c. of a 10-per-cent. solution suffices), and sufficient ferric chloride to colour the supernatant fluid yellowish-red. The mixture is then boiled, when all the iron separates out as phosphate and basic acetate. For those tissues yielding large quantities of phosphates, *e.g.*, liver, the amount of ferric chloride necessary is comparatively large, and some difficulty may be experienced with the filtration. The only possible help is obtained by using two or more filters. The washing of the precipitate must be carried out carefully, in spite of the considerable loss of time. Precipitation by ammonia may be carried out for the three subsequent repetitions. The precipitate is each time dissolved in the smallest possible quantity of acid.

After the united filtrates have been evaporated to dryness, the residue is again dissolved in water, and dilute hydrochloric acid added, about 6 c.c. of four times normal acid being usually sufficient. Sulphuretted hydrogen is then passed through the hot solution for at least half an hour, and it is then allowed to stand for a time, as the sulphides, which form in acid solution do not always readily separate out. The liquid is then filtered and the precipitate well washed with sulphuretted hydrogen water. The filtrate is again evaporated to dryness, re-dissolved in a little water on the water bath and then a solution of pure sodium hydrate is added in successive portions to the hot liquid, until no more ammonia comes off. Care should be taken to use as little sodium hydrate solution as possible, as every sample in the market contains small traces of iron. The nickel is thus precipitated in the form of the hydrate, and this is converted into nickel sesquioxide by the addition of 1 or 2 c.c. of bromine to the cold mixture. The nickel oxide is then collected by filtration, and after having been well washed, is dissolved in hydrochloric acid, and the solution evaporated to dryness to remove the excess of acid, and the residue re-dissolved in water with a faint trace of acid, in order to prevent the formation of basic salts. The solution is finally made up to a definite volume.

In the process of separation, no especial difficulties save the management of the voluminous iron precipitate, are met with as a rule. At times an insoluble residue is found on the filter paper when the oxide is dissolved. This is a trace of a sulphide of copper or another metal of this group, which has escaped precipitation by sulphuretted hydrogen in acid solution.

3. *Estimation.*—The usual method of quantitative estimation of nickel colorimetrically is carried out with ammonium sulphide, but it has been found that sharper results can be obtained by employing *a*-dimethylglyoxime $CH_3.C(N.OH).C(N.OH).CH_3$, which was recently shown by Tschugaeff to form a scarlet red compound with nickel in the presence of ammonia.*

For this purpose, a saturated solution of the reagent in absolute alcohol is prepared, this is diluted with water until a little of the compound separates out, and alcohol is then added until complete solution takes place. The fluid to be tested and a standard solution of nickel sulphate are placed in burettes. A measured quantity of the fluid is then run into a Nessler tube and to this 0·5 c.c. of a 10-per-cent. solution of ammonia and the same quantity of the dimethylglyoxime solution are added and the whole made up to 30 c.c. It is better first to add the ammonia to the nickel solution, then the dimethylglyoxime, and then allow the colour to develop before diluting up to the 30 c.c. mark. All the solutions must be cold. The fluid becomes coloured pinkish red, the depth of the coloration depending on the quantity of nickel present. The most convenient quantity to work with is about 0·08 to 0·01 milligramme. The colour is then compared with that produced by varying quantities of nickel from the standard solution. The determination is not complete until a quantity has been found, which gives a colour which is just too pink, and a second quantity the colour of which is just appreciably less pink, than the fluid to be tested. The quantity of nickel contained is then calculated as the amount midway between the two tubes. With a little practice, it is quite easy quickly to determine very small differences of colour. The estimation should be concluded as rapidly as is compatible with accuracy, as, after a short time, the nickel compound with dimethylglyoxime separates out of the coloured solution as a precipitate.

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* 'Deut. Chem. Ges. Ber.,' 1905, vol. 38, p. 2520.

The advantages of this method over the ammonium sulphide method are: (1) small traces of iron do not interfere with the final colour, nor with the sharpness of the method; (2) smaller quantities of nickel can be accurately estimated; and (3) it is easier to work in a bad light with the pink than with the brown colorimetric determination.

Dealing with the colorimetric test alone, with solutions of pure nickel sulphate, the smallest quantity which gives the reaction is 1/1000 milligramme. To detect such a small amount, the solution must be placed in the Nessler tube, then the ammonia and the solution of dimethylglyoxime added, when one can recognise the characteristic pink colour, and, lastly, the fluid is made up to 30 c.c.; on comparing this with distilled water, a faint but distinct difference is seen. 3/1000 milligramme gives a recognisable pink colour in 30 c.c. of fluid. Working with 0·07 milligramme, differences of 1/1000 milligramme can be recognised with a little practice. This represents a potential error of + or —0·7 per cent.

In test analyses, serum or blood with nickel sulphate added, the experimental error was kept as low as 2 per cent., using about 1 milligramme of nickel. For example, about 30 grammes of blood were placed in a crucible and 0·9 milligramme of nickel, in the form of the dissolved sulphate, was added. After ashing, extracting, removing the copper and iron groups, and precipitating the nickel in the form of the sesquioxide, etc., the final fluid was made up to 30 c.c.; 2 c.c. of this fluid were compared with varying quantities of a solution containing 0·01 milligramme of nickel per cubic centimetre. It was found that 5·9 c.c. gave a colour, which was just too pink, and 5·8 c.c., a colour, which was not pink enough, so that the 2 c.c. contained 0·0585 milligramme of nickel, and the whole solution 0·88 milligramme. This represents a loss of 0·02 in 0·9, or about 2 per cent.

Electrolysis is only to be preferred when large quantities of nickel are to be measured, while the method described above is intended for the recognition and measuring of quantities of nickel not exceeding a few milligrammes.

# *On Voges and Proskauer's Reaction for Certain Bacteria.*

By Arthur Harden, D.Sc., Ph.D.

(Communicated by Dr. C. J. Martin, F.R.S. Received December 5, 1905,—
Read February 1, 1906.)

(From the Chemical Laboratory Lister Institute.)

In 1898 Voges and Proskauer[*] described a new colour reaction which they had observed in the case of a bacillus, isolated by Voges and grown in a medium containing sugar. When potash was added and the tube allowed to stand for 24 hours or longer at room temperature, a beautiful fluorescent colour, somewhat similar to that of a dilute alcoholic solution of eosin, formed in the culture fluid, particularly at the open end of the tube exposed to the air. The reaction was found to be specific to the bacillus in question, and was not given by any of the other organisms isolated in the course of the investigation upon which they were engaged, nor by the *B. coli communis*, so that it afforded a most valuable means of differentiation for the inhabitants of the intestine. Durham[†] and How[‡] have also employed this reaction for the discrimination of intestinal bacteria, and MacConkey,[§] in confirmation of Durham, has found that out of a large number of bacteria which were tested only three gave the reaction, these being *B. lactis aerogenes* (Escherich), *B. capsulatus* (Pfeiffer), and *B. cloacæ* (Jordan).

The examination of the products formed by *B. lactis aerogenes* from glucose[||] has shown that acetylmethylcarbinol, $CH_3.CO.CH(OH).CH_3$, and 2·3-butyleneglycol, $CH_3.CH(OH).CH(OH).CH_3$, are both present in the medium in which this has been cultivated in the presence of glucose. The acetylmethylcarbinol has not as yet been isolated in the pure state, but is present in the aqueous distillate obtained by distilling the culture medium. This distillate and the glycol were, therefore, treated with caustic potash in order to ascertain whether either of them was the cause of the reaction just described. Neither of these substances produces the characteristic fluorescent coloration with potash alone, but when peptone water is also added, acetylmethylcarbinol gives the reaction after standing for about 24 hours, whilst the glycol does not react in this way even on standing. The coloration was produced in the characteristic manner described by

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[*] 'Zeitschr. f. Hyg.,' 1898, vol. 28, p. 20.

[†] 'Journ. of Experimental Medicine,' 1900—1901, p. 354.

[‡] 'Centralbl. f. Bakter.,' 1904, vol. 36, p. 484.

[§] 'Journ. of Hyg.,' 1905, vol. 5, 349.

[||] Harden and Walpole.

Voges and Proskauer, commencing at the open end of the tube exposed to the air. This suggests oxidation as a factor in the phenomenon, and as acetylmethylcarbinol is very readily converted by oxidation into diacetyl, $CH_3.CO.CO.CH_3$, this substance was tested. Diacetyl yields the fluorescent red coloration with peptone water and caustic potash in a few minutes, and by its aid a much greater depth of colour can be obtained than that observed with bacterial cultures.

Voges and Proskauer's reaction, therefore, appears to be due to acetylmethylcarbinol, which is formed by the action of the bacteria on the glucose of the medium. In the presence of potash and air this is oxidised to diacetyl, which then reacts with some constituent of the peptone water. That diacetyl is the active substance and not *p*-xyloquinone, $C_6H_2O_2(CH_3)_2$, which is readily formed from it by the action of alkalis, is shown by the fact that if the diacetyl be allowed to stand for some time with potash solution, and peptone water be then added, no reaction occurs.

*B. cloacæ* (Jordan), which gives Voges and Proskauer's reaction, was also found to yield acetylmethylcarbinol, which was recognised by its power of reducing Fehling's solution in the cold and of yielding the characteristic phenylosazone of diacetyl with phenylhydrazine.

Acetylmethylcarbinol has also been observed as a product of the action of certain other bacteria on glucose. Thus, Grimbert* found that it is produced by *B. tartricus*, and Desmots† that it is also formed by the various bacilli of the mesentericus group and by *B. subtilis* and *Tyrothrix tenuis*. These bacteria should, therefore, give Voges and Proskauer's reaction, and, as a matter of fact, *B. mesentericus fuscus*, the only one which has so far been examined, gives the reaction quite characteristically when grown in peptone water containing 2 per cent. of glucose.

A number of other bacteria are being examined and attempts are also being made to ascertain what constituent of the peptone water it is that reacts with the diacetyl.

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* 'Compt. Rend.,' 1901, vol. 132, p. 706.

† 'Compt. Rend.,' 1904, vol. 138, p. 581.

# *A Further Communication on the Specificity and Action* in Vitro *of Gastrotoxin.*

By Charles Bolton, M.D., Research Scholar of the Grocers' Company.

(Communicated by Professor Sidney Martin, F.R.S. Received January 25,—Read February, 1, 1906.)

(From the Pathological Laboratory, University College, London.)

[Plates 16 and 17.]

In July, 1904, I laid before the Royal Society a preliminary communication on the production of a gastrotoxic serum. In that communication I stated that the serum, obtained by the injection of the mucous membrane of the stomach of the guinea-pig into the rabbit, was not specific in the true sense of the word; and further, that I was unable to demonstrate any effect of the gastrotoxin upon the gastric glands of the guinea-pig *in vitro*, although necrosis and ulceration of the mucous membrane of the stomach were produced by injection of the serum into the living animal.

By means of more extensive experiments and improved methods I have obtained confirmatory evidence that the action of the serum is not truly specific, and have also been able to demonstrate that a definite effect upon the gastric cells is produced *in vitro*. I have also succeeded in preparing a gastrotoxic serum by injecting the fresh mucous membrane of human stomach into the rabbit. The present communication is therefore intended as a continuation of my former one.

The subject will be discussed under the following headings:—

I. ACTION *in Vitro*—
  1. Hæmolytic action.
  2. Action upon the gastric granules (agglutination).
  3. Action upon the soluble proteids of the cells (precipitation).
  4. Action upon the intact gastric cells (lysis).

II. SPECIFICITY OF GASTROTOXIN—
  1. *Power of Different Cells to Render the Serum Inactive.*
    Experiments *in vivo.*
    Experiments *in vitro.*
  2. *Comparison with Entero- and Hepatotoxin and Hæmolysin.*
    Experiments *in vivo.*
    Experiments *in vitro.*

III. PRODUCTION OF HUMAN GASTROTOXIN.

IV. GENERAL CONCLUSIONS.

## I. ACTION *in Vitro.*

In my first communication I stated that the blood serum of the rabbit injected with stomach cells washed free from blood became more highly hæmolytic for guinea-pig's red-blood corpuscles than it was previous to injection. I have recently been able to demonstrate that two distinct hæmolysins are produced during the process of immunisation.

The gastrotoxin has likewise the power of producing marked changes in the soluble proteids, and also in the protoplasmic granules of the gastric cells. It further brings about slight though definite changes in the intact cells themselves.

### 1. *Hæmolytic Action.*

*Method.*—In obtaining the guinea-pig's blood corpuscles to test the hæmolytic power of the immunised rabbit's serum, the blood is whipped and centrifugalised, the serum then pipetted off and the corpuscles washed in 0·86-per-cent. salt solution several times. A 5-per-cent. suspension of the corpuscles in salt solution is used for testing the serum.

A known quantity, usually 1 c.c., of this suspension of corpuscles is mixed with diminishing amounts of the serum in a series of test-tubes, the volume of fluid in each tube being made up to the same amount with salt solution. The tubes are placed in the incubator for one hour, and then in the ice chamber till the following morning, when the exact point at which the corpuscles are completely dissolved can be determined with ease.

As an example, the details of the following experiment which was made to determine the normal hæmolytic power of a rabbit's blood for guinea-pig's corpuscles are given:—

| Corpuscles (5 per cent. suspension). | Normal rabbit's serum. | | Salt solution. | After 1 hour's incubation and ice chamber. |
|---|---|---|---|---|
| c.c. | | c.c. | c.c. | |
| 1 | | 2·75 | 0·25 | Complete solution. |
| 1 | | 2·5 | 0·5 | " |
| 1 | | 2·25 | 0·75 | " |
| 1 | | 2 | 1 | " |
| 1 | | 1·75 | 1·25 | Almost complete. |
| 1 | | 1·5 | 1·5 | Incomplete. |
| 1 | | 1·25 | 1·75 | " |
| 1 | | 1 | 2 | " |
| 1 | | 0·75 | 2·25 | " |
| 1 | | 0·5 | 2·5 | " |
| 1 | | 0·25 | 2·75 | Fluid above corpuscles uniformly tinted. |
| 1 | Diluted 10 times. | 0·1 | 2 | Fluid tinted to diminishing heights above corpuscles. |
| 1 | | 0·075 | 2·25 | |
| 1 | | 0·05 | 2·5 | |
| 1 | | 0·025 | 2·75 | |
| 1 | Diluted 100 times. | 0·01 | 2 | |
| 1 | | 0·0075 | 2·25 | No laking. |
| 1 | | 0·005 | 2·5 | " |
| 1 | | 0·0025 | 2·75 | " |

In the test-tube containing 2 c.c. serum there was complete solution of the corpuscles, and in the test-tube containing 0·01 c.c. serum there was a trace of hæmoglobin diffused in the clear fluid just above the deposited corpuscles.

From the experiment it was therefore found that 2 c.c. serum of this rabbit would completely lake 1 c.c. of a 5-per-cent. suspension of guinea-pig's corpuscles, and that a dilution of the serum of 1 in 400 was the greatest which would produce any solution at all.

It is not reliable to estimate the hæmolytic power solely by finding the greatest dilution in which any solution will occur, or in other words the vanishing point of hæmolysis, because this vanishing point may occur in higher dilutions in the case of a weaker serum than in the case of a stronger serum. What is the exact reason for this phenomenon does not appear to be at all clear. Gay (1) has, however, recently shown that in the case of high dilutions the activity of the complement may be completely inhibited. Bashford (2) has suggested that in the higher dilutions hæmolysis is interfered with by agglutination of the red corpuscles.

The hæmolytic power of most rabbits is fairly constant, but as they vary somewhat within small limits, I examine the hæmolytic power of each rabbit's blood before injection. Care must be taken to use exactly the same dilutions when the serum is subsequently tested, as the amount of dilution affects the hæmolytic power of the serum.

*Hæmolysis.*—A few days after the first injection of guinea-pig's stomach cells into the peritoneal cavity of the rabbit, the hæmolytic power of the rabbit's serum for guinea-pig's red corpuscles is found to have considerably increased. In one case before injection 2·25 c.c. serum were necessary to completely dissolve 1 c.c. of a 5-per-cent. suspension of corpuscles; seven days after the injection 0·75 c.c. serum would dissolve the same amount of corpuscles.

This first increase of hæmolysin is a true increase of the natural hæmolysin of the rabbit, because its action like that of the natural hæmolysin is destroyed by heat, and is not restored on adding normal guinea-pig's serum. In other words, guinea-pig's complement will reactivate neither.

At this early stage a slight amount of laking, varying in extent in different cases, may be seen on reactivating the heated serum with guinea-pig's complement, but this laking has never been to any degree extensive enough to account for the increase of hæmolysis. In the above experiment, 21 days after the first injection, it was found that 0·5 c.c. serum would completely dissolve 1 c.c. suspension of corpuscles, but on heating 2·5 c.c. immune serum to 55° C., and complementing with 0·25 c.c. guinea-pig's serum, only the faintest trace of hæmoglobin was seen to be diffused in the fluid immediately above the deposited corpuscles. After the second injection, however, it is found that guinea-pig's serum will reactivate the heated immune serum to a considerable extent. In the above experiment six days after the second injection 0·5 c.c. heated immune serum, on being reactivated by 0·25 c.c. guinea-pig's serum, completely dissolved the test amount of corpuscles. I have confirmed this experiment several times, and to my mind it conclusively points to the presence of two distinct immune bodies. (1) An increase of the normal hæmolysin of the rabbit which *is not* complemented by guinea-pig's serum. (2) A newly-formed and therefore artificial hæmolysin which *is* complemented by guinea-pig's serum.

This has an important bearing upon the hypothesis of the multiplicity of immune bodies, which is upheld by Ehrlich and Morgenroth (3), but is denied by other observers, notably Muir and Browning (4), and Gay (5) working in the Pasteur Institute. Agglutination of the red corpuscles also occurs.

### 2. *Action upon the Protoplasmic Granules of the Gastric Cells.*

*Method.*—The method which I employ in order to demonstrate this action is an imitation of that described for hæmolysin.

The mucous membrane of a guinea-pig's stomach is first washed free from blood by sterilised salt solution, which is made to flow through a canula introduced into the thoracic aorta, the stream issuing from the inferior vena cava. It is then scraped off, and the pulp ground up in a glass mortar. An emulsion is made with salt solution and centrifugalised for five minutes at a low speed. The supernatant fluid on being pipetted off is found to contain in suspension innumerable large and small protoplasmic granules. The granules are separated from the albuminous fluid in which they float by centrifugalisation at a high speed, and repeatedly washing until the washings give no precipitate with potassium ferrocyanide and acetic acid.

The final suspension of granules in saline solution, which is to be used, must be well agitated so as to free all the granules, and before use it must be slowly centrifugalised to ultimately remove any masses of granules or pieces of tissue which happen to be present.

A series of test-tubes is prepared, each tube containing 2 c.c. of the gastrotoxic serum in increasing dilutions. The first tube contains undiluted serum, and the remainder dilutions from 1 in 5 to 1 in 320. To the contents of each tube three to five drops, according to concentration of the suspension of granules in salt solution, are added.

A control of normal saline is prepared, and also a control in which

0·5 c.c. of the washings is added to 2 c.c. serum, so as to eliminate any possibility of a precipitate of albumin being thrown down from the fluid, in which the granules are suspended, by the immune serum.

The tubes are incubated for four or five hours and then examined by the naked eye and also microscopically.

*Agglutination.*—A very fine deposit consisting of agglutinated granules is seen, sometimes only with a lens, at the bottom of the tubes up to a certain dilution which varies according to the strength of the serum. The supernatant fluid contains granules in all stages of agglutination, and if the tubes are allowed to stand in the ice chamber till the next day, all the agglutinated granules are found to have settled to the bottom.

The reading of the tubes is taken at the end of four or five hours' incubation, and controlled by that taken on the following day, both macro- and microscopically. The reason for taking the reading twice is because bacteria are liable to be found in the fluid at the end of 24 hours, since the scraped mucous membrane cannot be sterilised by heat.

The saline control shows no such deposit or agglutination, and the control containing the washings shows no precipitate or deposit. The blood of a normal rabbit shows no deposit and no agglutination, and can therefore be used as a control.

The amount of deposit is just sufficient to be accounted for by the subsidence of the granules, and on shaking it up the agglutinated granules pass into suspension, forming a delicate precipitate. Vigorous shaking disentangles the granules, and the appearance of the solution becomes the same as it was when the granules were first added.

It is thus evident that the serum possesses an action upon the granules themselves, and that this action is similar to that of the bacterial agglutinins. The agglutinin appears in the rabbit's blood in small amounts about 14 days after the first injection, and can be quite easily recognised after a second injection.

*Effects of Heat.*—After exposure to a temperature of 58° to 60° for half an hour the serum agglutinates gastric granules in as high a dilution as it does when unheated. In this property of resisting heat the agglutinin resembles those of bacterial origin.

### 3. *Action upon the Soluble Proteids of the Gastric Cells.*

*Method.*—The solution of proteid is prepared by grinding up the mucous membrane of the guinea-pig's stomach, previously washed free from blood, as described above, in a glass mortar with normal salt solution.

The emulsion is centrifugalised in order to get rid of most of the solid matter, but it is not possible to completely free such a solution from protoplasmic granules by cenrtifugalisation alone. The solution is filtered through a Berkefeld filter. The resulting filtrate, which is a perfectly clear solution like water, gives a precipitate with heat, ferrocyanide of potassium, and acetic acid, and other precipitants of proteids.

The experiment is done in the same way as that described for the agglutination test. The gastrotoxic serum is diluted and placed in a series of test-tubes, and to the contents of each test-tube 0·5 c.c., or even only two or three drops of the proteid solution, are added. The tubes are placed in the incubator for four or five hours. Controls of normal rabbit's serum and salt solution are also prepared.

*Precipitation.*—At the end of one hour's incubation a fine precipitate has commenced to form in the solution. This precipitate becomes coarser in appearance, and at the end of about four hours has settled to the bottom of the tube in considerable quantity, forming a deposit. Flakes of precipitate can still be seen floating about in the otherwise perfectly clear supernatant fluid. Under the microscope this precipitate is seen to consist of amorphous-looking masses. If the tubes are placed in the ice-box until the next day the whole of the precipitate will be found to have settled to the bottom, leaving the supernatant fluid perfectly clear.

As in the case of the agglutination test, I record the result of the experiment both at the end of four or five hours, and also on the following day, although in this case one can exclude the presence of bacteria, since the tubes have been sterilised and plugged with cotton wool, and the gastric solution filtered.

The control tube containing normal rabbit's serum, and that containing salt solution, show no precipitate after incubation. It is thus perfectly clear that the gastrotoxic serum acts chemically upon the soluble proteids of the guinea-pig's gastric cells, producing an insoluble compound. This precipitin appears in the serum about the same time as the agglutinin.

*Effects of Heat.*—As in the case of the agglutinin, exposure to a temperature of 58° to 60° C. for half an hour does not in the least diminish the power of the serum to precipitate the soluble proteid, since precipitation occurs in as high a dilution of the heated serum as it does in the unheated serum.

It is well known that the effects of heat upon the action of precipitins varies considerably; some are easily affected, whilst others are resistant.

*Action upon Guinea-Pig's Blood Serum.*—The experiment is performed in exactly the same manner as described above. The guinea-pig's serum is diluted 10 or more times, and 0·5 c.c. added to 2 c.c. gastrotoxic serum.

The mixture is incubated, and at the end of four hours it is seen that a similar precipitate has formed. The gastrotoxic serum may precipitate the serum in as high a dilution as it does the proteid of the gastric cells. The gastrotoxin does not therefore act exclusively upon the proteid of the gastric cells.

### 4. *Action upon the Intact Gastric Cells.*

*Method.*—A guinea-pig's stomach is washed quite free from blood before removal from the body, as described above. The superficial portion of the mucous membrane is gently scraped off with a knife and suspended in a few cubic centimetres salt solution. The test-tube is now very carefully shaken up for a few seconds and allowed to stand for about 10 minutes, at the expiration of which time the contents will have separated into two portions :—

1. A milky fluid.
2. Small pieces of mucous membrane, which either float on the top of the fluid or settle to the bottom.

The milky fluid is the portion used. It is pipetted off, and on microscopic examination is found to contain in suspension free cells, masses of cells, and fragments of glands, together with broken-up cells, free nuclei, and protoplasmic granules.

It is quite easy to separate the cells, because on slow centrifulgalisation the cells and fragments of glands sink to the bottom, but the granules and nuclei remain in suspension.

The cells are washed to clear away the granules and soluble proteid. A suspension of cells, masses of cells, and fragments of glands in salt solution is thus obtained.

The free oxyntic cells are large oval structures with well-defined granules, but the central cells, which are of a more delicate structure and contain finer granules, tend to cling together in masses and are easily broken up if too vigorous shaking is employed.

In doing an experiment three or four drops of suspension of cells are placed in 2 c.c. gastrotoxic serum in a test-tube, and the mixture incubated for four or five hours. Controls are prepared with normal rabbit's serum and also salt solution.

*Lysis.*—The deposit which has formed in the tube is examined microscopically. I have done a large number of these experiments, and always with the same results. I have never observed either solution or agglutination of the cells, such as has been described in the case of other cytotoxins; in fact, the cells in the serum are much more separated than those in the salt solution, as the latter tend to stick together by reason of the mucus, which it is impossible to clear away completely.

The cells in the salt solution are quite normal in appearance, but the cells which have been exposed to the action of the gastrotoxic serum have become more or less hyaline.

The oxyntic cells are not so much affected as the central cells, the masses of which appear like pieces of floating glass. The granules are obscured, and many of the cells look like shadows. The nuclei can usually be seen except when the cells are massed together.

The cells are examined in the fresh state, as this seems to me to be the best for practical purposes. I have also stained them with watery solutions of methyl green, picrocarmine, and safranin, but this does not materially assist. The stained cells which are affected look like pieces of coloured glass.

This effect of the gastrotoxic serum appears later than those described above, and is not observed until after the expiration of about five weeks after the first injection, four or five injections having been given in the meanwhile. The effect begins to pass off between three and four months after the first injection, the animal receiving injections at regular intervals.

The serum of a normal rabbit differs in no respect in its behaviour towards the gastric cells from salt solution.

*Effects of Heat.*—A temperature of 55° C. maintained for half an hour does not destroy the action of the serum upon the gastric cells. It may be weakened, but only to a slight degree. This result points to the conclusion that a complement exists in the cells themselves, if the action is due to a cytolysin of the same construction as a hæmolysin. Such endocellular complements have of course been described before.

I have heated the stomach to 55° C. for half an hour before obtaining the cells, and have found that in this case the serum produces no effect upon them. This experiment of course proves nothing, because it is probable that the vitality of the cells is destroyed by exposure to this temperature, since cell globulin coagulates at 48° to 50° C.

*Removal of Gastrolytic Factor.*—By saturating the serum with washed gastric cells of the guinea-pig and allowing the mixture to stand for one hour, the gastrolytic factor is removed and the action of the serum upon the cells is destroyed. No change is visible in the cells which have been used to saturate the serum in the space of one hour, and, since they remove the gastrolysin, it is evident that the latter becomes anchored to the gastric cells preparatory to acting upon them. This brings the gastrolysin into line with a hæmolysin in this respect.

## II. SPECIFICITY OF GASTROTOXIN.

If this serum be strictly specific for the stomach cells of the guinea-pig, it should possess chemical affinities for the stomach cells alone and for no other tissues of the body. On injection into the guinea-pig it would then produce lesions limited to the stomach, and would also be rendered inactive by mixture with stomach cells, which would combine with the poisonous substance in the serum and take it out of solution. Further, other tissues of the body should not possess chemical affinities corresponding to those of the stomach cells and, therefore, would not render the serum inactive by mixture with it, and sera formed against those tissues would not produce lesions in the stomach on injection.

The specificity has therefore been tested by comparing the relative power of different cells of the body of the guinea-pig to render the serum inactive, and also by comparing the effects of the serum with those of a hepatotoxic, an enterotoxic, and a hæmolytic serum obtained by injecting blood.

The experiments have been conducted *in vivo* and *in vitro.*

### 1. *Power of Different Cells to Render the Serum Inactive.*

In my former communication I gave the results of a few experiments. I have since examined this question more extensively and although the subject is far from being completed yet I will here give the results of my further investigations.

In mixing the various cells with the serum to be examined, care must be taken that enough cells are present to saturate the serum otherwise when they settle to the bottom of the tube a portion of the serum is left unexposed to their action. The cells obtained by scraping the mucous membrane of one guinea-pig's stomach are enough to saturate 4 c.c. serum, but the resulting mixture will not yield 4 c.c. serum back. For a reliable experiment it is necessary to inject at least 10 c.c. serum and, therefore, to obtain this I take 12 c.c. serum and mix with it the mucous membrane of three guinea-pigs' stomachs. After centrifugalisation it is quite easy to obtain 10 c.c. of treated serum.

The cells of the mucous membrane of the small intestine are obtained in the same way as those of the stomach.

The liver is pounded up and passed through a tea strainer in order to prepare it. In all cases the blood is washed out of the organ in question before it is removed from the body.

If the serum is examined *in vivo* after such treatment a control animal is, in each case, injected with the untreated serum; if it is examined *in vitro* controls are likewise prepared in all cases.

All the experiments have invariably been carried through to a finish on the same day, and all the tissues and sera used were always obtained fresh on that day. In testing the hæmolytic power of the treated serum equal weights of the various organs were previously mixed with equal volumes of the serum. The cells were allowed to stand in contact with the serum for one hour at laboratory temperature, except in the case of some of the *in vitro* experiments, when the tubes were placed in the incubator.

*Experiments* in Vivo. *Mixture with Stomach Cells.*—Four experiments have been done, and in each case with the same result. The gastrotoxin was in all the cases completely removed by the stomach cells, and the resulting serum produced no lesions in the stomach at all. The control animals in all cases showed the usual lesions (see Plate 16, fig. 1).

*Mixture with Intestine Cells.*—In four cases the intestine cells failed to destroy the action of the serum, the lesions produced by the treated serum being as extensive as those produced by the untreated serum (see Plate 16, fig. 2).

In three cases the action was destroyed, but the lesions in the three control animals were so slight that the toxicity of the serum must have been very low.

*Mixture with Liver Cells.*—In four cases the liver cells failed to destroy the action of the serum, but in each case there was a weakening of the power of the gastrotoxin, judging by a comparison with the effects produced in the control animals (see Plate 16, fig. 3).

In one case complete removal of the gastrotoxin resulted, and in this case the latter was of low toxic value, judging by the lesions produced in the control animal (see Plate 16, fig. 4).

*Mixture with Red Blood Corpuscles.*—In three experiments the action of the gastrotoxin was unaffected, and in a fourth the lesion was less extensive than that in the control animal (see Plate 17, fig. 5).

The results of these experiments clearly demonstrate that other organs of the body besides the stomach have tissue affinities for, at all events, *some* of the constituents of this serum, and that they can, if not invariably destroy its action, at any rate weaken it. On the other hand, the stomach is the only organ of the body which can invariably and with uniform certainty destroy the action of the gastrotoxin. The serum is thus not, strictly speaking, specific, although lesions are not produced in other organs than the stomach by it as a rule. It may be, however, that one of the constituents of this complex serum is specific for the stomach to a great extent.

These experiments also illustrate the importance of an organ's ability to take up a poison and render it inactive without being itself affected by it. They likewise explain why large doses of the serum are necessary to produce the stomach lesions, since a large part of this serum must be rendered inactive by different organs of the body.

*Experiments* in Vitro. *Hæmolysin. Mixture with Stomach Cells of Guinea-pig.*—After treatment with stomach cells the serum shows as high a degree of hæmolytic power as it did before such treatment. It may be higher. At first sight this result appears to be remarkable, namely, that a cell will not remove a side chain that is thrown off in response to its injection. In other words, that side chains may be thrown off which have no affinity at all for the cells against which they are thrown off.

I have obtained further evidence of the same principle in the case of the gastrotoxin formed against guinea-pig's stomach cells by injection of the rabbit's stomach cells into the rabbit. The rabbit's stomach cells will not remove this gastrotoxin from the serum, and therefore, whether one supposes that the cytophilic affinity of the amboceptor for the rabbit's stomach cell is or is not saturated by an anti-immune body, the fact remains that the rabbit's stomach cell has no affinity for the side chain which is active against the guinea-pig's cell, and which has been thrown off in response to injection of rabbit's stomach cell. Similarly rabbit's stomach cells will not remove the gastrotoxin from guinea-pig-rabbit gastrotoxic serum (see Plate 17, fig. 6).

It seems to me most likely that when a cell is absorbed side chains having an especial affinity for that cell, and which are used in destroying it, are set free and that other side chains having less affinity for it are set free in diminished amount, and also side chains having no affinity whatever for it are set free in smallest amount. In other words, the absorbing cell throws off most of the varieties of side chains or chemical affinities of which it is possessed, the number of each being directly determined by the amount of stimulation given to the particular chemical affinity involved.

The fact that the stomach cells will not absorb the hæmolysin is important from another point of view. I shall show later that the action of a hæmolysin, whatever its origin, is directed especially against the stomach, and also that lesions due to its action may be limited to the stomach. Now if other organs of the body have the power of destroying the hæmolysin without themselves being affected, whilst the stomach cells will not absorb it, the result naturally follows that the hæmolysin will be free to act as it may in the capillaries of the stomach, and therefore will produce lesions.

*Mixture with Liver Cells, Intestine Cells, and Red-Blood Corpuscles.*—Each of these three varieties of cells has the power of removing the hæmolysin from the serum, and therefore of rendering it incapable of dissolving the red-blood corpuscles of the guinea-pig *in vitro.* Occasionally, especially in the case of the liver, a slight amount of diffused hæmoglobin may be seen above the settled corpuscles.

These experiments clearly indicate that *although the hæmolytic factor of the gastrotoxic serum may be of great importance in assisting to produce the stomach lesions* in vivo, *yet it is not the only one.* The reasons for this statement are, that previous mixture with stomach cells will deprive the serum of its action *in vivo,* but will not prevent its laking blood corpuscles *in vitro ;* that previous mixture with liver cells, intestine cells, or red-blood corpuscles, although it deprives the serum of its power to lake blood corpuscles *in vitro,* will not with any degree of uniform certainty completely prevent its action *in vivo.*

*Lysin.*—I have not yet attempted to compare the gastrolytic strengths of two sera by determining the highest dilutions in which any action is apparent, and therefore cannot say whether or not the action in any given case is diminished. So far as my results go, however, they appear to indicate that, after exposure of the serum to gastric cells, its action upon such cells is destroyed, but that after exposure to liver and intestine cells and blood corpuscles the serum still produces changes in gastric cells *in vitro.*

These *in vitro* experiments, so far as they go, point to the same conclusion as the *in vivo* experiments—namely, that this gastric cytotoxin is not truly specific, although one or more of the bodies contained in it may be so, and that the protoplasmic poisons constituting it have a greater affinity for gastric cells than for the cells of other organs of the body.

### 2. *Comparison with Hepato- and Enterotoxic Sera and Hæmolysin.*

The hepatotoxin and enterotoxin were respectively prepared by injecting the rabbit with the washed and prepared cells of the liver and intestine of the guinea-pig. The hæmolysin was obtained by injecting red-blood corpuscles.

*Experiments* in Vivo.—Each of these sera produces hæmolytic lesions in the stomach, leading to destruction of the mucous membrane, the microscopic condition very closely simulating that due to gastrotoxin (see Plate 17, fig. 7).

They are more uncertain in their action upon the stomach, however, and this action is liable to be not so strictly limited to the stomach as that of gastrotoxin. The action of hæmolytic serum was described in my previous communication.

*Experiments* in Vitro. *Hæmolytic Power.*—Both hepato- and enterotoxin possess the power of dissolving red-blood corpuscles to about the same degree as gastrotoxin. They also agglutinate these cells.

*Agglutination and Precipitation.*—The gastrotoxic serum produces similar effects upon emulsions of liver and intestine granules to those described in the case of emulsions of stomach granules.

Hepato- and enterotoxin not only act upon emulsions of liver and intestine granules, but they also act upon emulsions of stomach granules. Hæmolysin obtained by injecting blood has no more power of acting upon these emulsions than normal rabbit's blood has.

*Lysin.*—Up to the present no definite hyaline transformation of gastric cells has been demonstrated as the result of the action of hepato- or enterotoxin or hæmolysin, and gastrotoxin does not appear to act upon the intact liver or intestine cells.

*Removal of the Immune Body by Different Cells.*—Only one or two experiments of this nature have been done up to the present. In the case of a rabbit which had been immunised against the red-blood corpuscles of the guinea-pig, mixture with stomach cells entirely failed to remove the hæmolysin and stomach lesions resulted on the injection of the serum (see Plate 17, fig. 8). Mixture with liver and intestine cells, however, rendered the same serum less powerful than before.

In the case of an enterotoxic rabbit, mixture of the serum with either stomach cells or intestine cells effected complete removal of the amboceptor.

*In vitro*, stomach cells completely fail to remove the hæmolytic factor from enterotoxic or hepatotoxic serum, as was previously observed in the case of gastrotoxic serum. Liver and intestine cells remove the hæmolytic factor from entero- and hepatotoxin as they do in the case of the gastrotoxin.

The few experiments that have been made with hepato- and enterotoxin therefore confirm the view that gastrotoxin is not strictly specific.

## III. PRODUCTION OF HUMAN GASTROTOXIN.

Nine rabbits have been immunised against fresh human stomach mucous membrane. Four died from septic infection, the remaining five gave positive results.

This is not quite such an easy matter as in the case of the guinea-pig's stomach, because the supply of human stomach is not constant, and three weeks or a month may elapse without an opportunity for obtaining the mucous membrane offering itself. In addition to this the stomach cannot be used immediately after death, although on one or two occasions I have been fortunate enough to obtain some from operation cases, and it is impossible to obtain the stomach free from blood.

The normal serum of the rabbit is slightly hæmolytic for human blood corpuscles, but does not produce any effect upon emulsions of human stomach granules.

I have succeeded in showing that the sera of five rabbits so immunised became highly hæmolytic for human blood corpuscles, and in the one case in which I tried it, solution of the corpuscles of the monkey also occurred. The sera also agglutinated and precipitated emulsions of human gastric granules, and in one case those of the monkey also. Whether hyaline changes are produced in the cells I have not yet determined.

## IV. GENERAL CONCLUSIONS.

The gastric cytotoxin formed in the blood of an animal in response to the injection of gastric cells thus appears to be a complex body. After a single injection there is a great increase in the hæmolysin normally occurring in the animal's blood, and at the same time there is found a new hæmolytic immune body which is not normally present in the animal. The latter is present in considerable amount after the second injection. The gastrotoxin also agglutinates red-blood corpuscles. Closely associated with the appearance of this artificial hæmolytic immune body is that of an agglutinin which acts upon the gastric granules, and also that of a precipitin which acts upon the soluble proteids of the gastric cells.

By repeating the injections these substances are found to be present in the blood for several months. Whether they are one and the same or distinct bodies I have not yet proved.

After several injections, and not less than about five weeks from the first, a further substance appears in the blood, which possesses an action upon the intact gastric cells. In spite of repeated injections, this substance disappears from the blood in about four months. It is probably of the same nature as a hæmolysin, but this point requires proof.

The hæmolytic factor is only active against blood. The actions of the agglutinin and precipitin are not confined to the constituents of the gastric cells, but extend to other proteids of the body. Whether there are separate agglutinins and precipitins for different proteids, or whether the same substances act upon all proteids, has not been determined; at all events, if the same bodies are concerned in all cases, their action upon the proteids of the stomach cells is probably greater than that upon other proteids.

Whether the gastrolysin itself is truly specific remains to be proved.

The few experiments that have been undertaken in the case of the human stomach indicate that the human gastric cytotoxin is identical in constitution with that of the lower animals.

*Note.*—The term "Hæmolytic lesions" is used in this paper to signify the hæmorrhages which are produced by the injection of a hæmolytic serum; this does not however imply that such hæmorrhages are directly caused by the factor in the serum which brings about solution of the red blood corpuscles.

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2. Bashford, 'Journal of Hygiene,' vol. 4, 1904, No. 1, p. 40.
3. Ehrlich and Morgenroth, 'Berl. Klin. Wehschr.,' 1901, p. 569—598, and 1900, p. 681.
4. Muir and Browning, 'Roy. Soc. Proc.,' vol. 74, 1904, p. 298.
5. Gay, *loc. cit.*

## DESCRIPTION OF PLATES.

### PLATE 16.

FIG. 1.—Illustrates the removal of the gastrotoxin from the serum by treatment with guinea-pig's stomach cells previous to its injection.

*Upper Stomach.*—From a guinea-pig injected with gastrotoxic serum. Necrosis of the mucous membrane has therefore resulted.

*Lower Stomach.*—From a guinea-pig injected with the same dose of the same serum previously treated with stomach cells. No lesion has resulted; the stomach cells have removed the gastrotoxin from the serum by combining with it.

FIG. 2.—Stomach of a guinea-pig which was injected with gastrotoxic serum previously treated with guinea-pig's small intestine cells. The gastrotoxin has not been removed from the serum by the cells, and the stomach therefore shows necrosis of the mucous membrane.

FIG. 3.—Stomach of a guinea-pig which was injected with gastrotoxic serum previously treated with guinea-pig's liver cells. The gastrotoxin has not been removed from the serum by the liver cells, and the stomach therefore shows a patch of necrosis of the mucous membrane. The action of the serum has, however, been weakened.

FIG. 4.—*The lower stomach* is that of a guinea-pig which was injected with gastrotoxic serum previously treated with guinea-pig's liver cells. The gastrotoxin has been removed in this case by the liver cells, and the stomach therefore shows no lesion.

*The upper stomach* is from the control animal, which was injected with untreated serum, and shows lesions which are relatively slight.

### PLATE 17.

FIG. 5.—Illustrates the fact that guinea-pig's red blood corpuscles will not remove the gastrotoxin from the serum by combining with it.

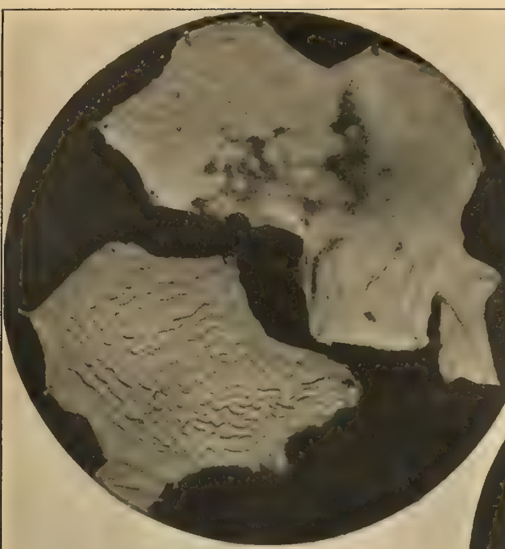

Fig. 1.

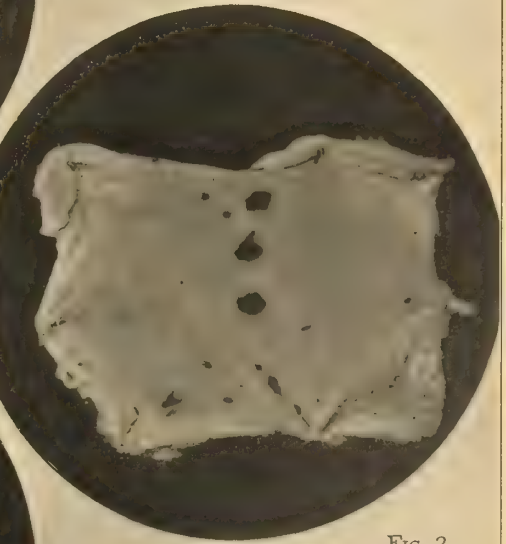

Fig. 2.

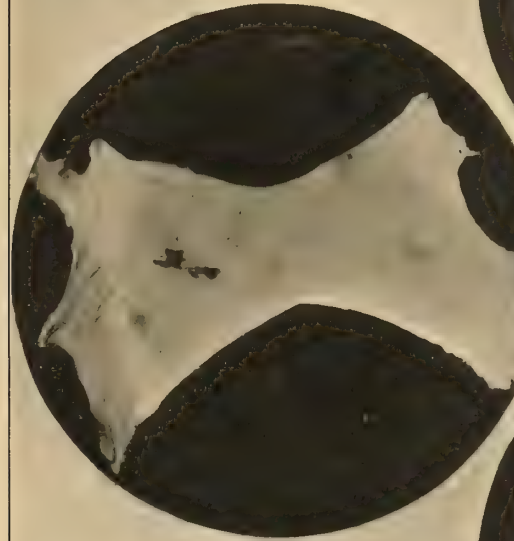

Fig. 3.

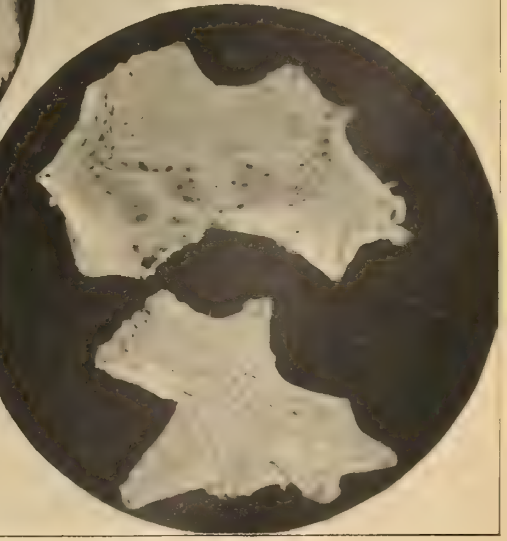

Fig. 4.

[*Photographs by E. S. Worrall.*]

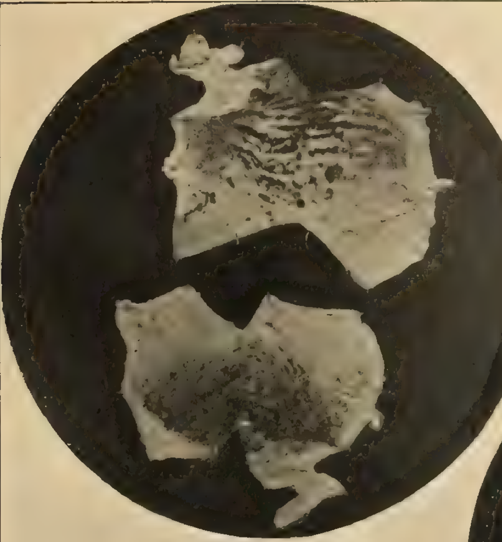

FIG. 5.

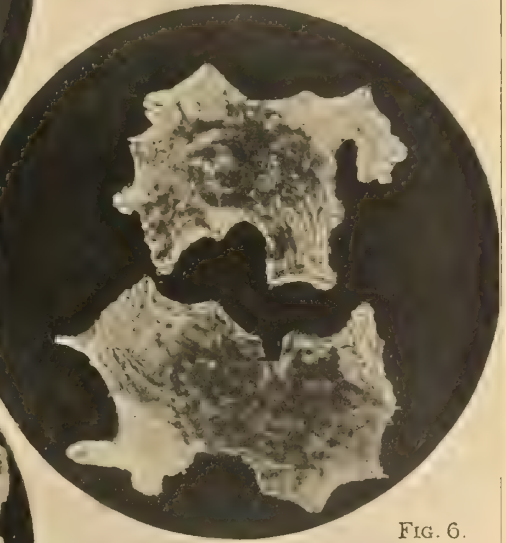

FIG. 6.

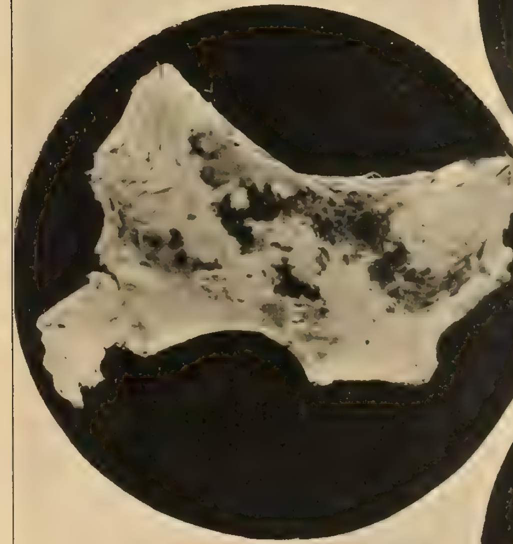

FIG. 7.

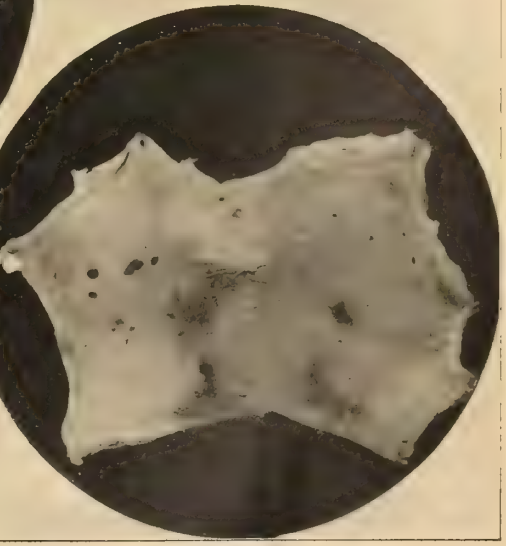

FIG. 8.

[*Photographs by E. S. Worrall.*]

*Upper Stomach.*—From a guinea-pig which was injected with gastrotoxic serum previously treated with blood corpuscles. Necrosis of the mucous membrane has resulted, because red blood corpuscles will not combine with the gastrotoxin.

*Lower Stomach.*—From the control animal, which was injected with the same dose of untreated gastrotoxic serum, necrosis of the mucous membrane has therefore resulted.

FIG. 6.—Illustrates the fact that rabbit's stomach cells will not remove the gastrotoxin from guinea-pig-rabbit gastrotoxic serum.

*Upper Stomach.*—From the control animal, which was injected with untreated serum, and therefore shows extensive necrosis and hæmorrhage.

*Lower Stomach.*—From a guinea-pig which was injected with gastrotoxic serum, previously treated with rabbit's stomach cells. The cells have failed to remove the gastrotoxin from the solution, and lesions similar to those of the control animal have resulted.

FIG. 7.—Illustrates the hæmolytic lesions which are produced in a guinea-pig's stomach by the injection of a guinea-pig-rabbit entrotoxic serum. As seen here, the lesions are indistinguishable from those produced by gastrotoxic serum.

FIG. 8.—Illustrates the fact that guinea-pig's stomach cells will not remove the hæmolysin from guinea-pig-rabbit hæmolytic serum (obtained by injecting red blood corpuscles). Hæmolytic patches are seen in the stomach, which is that of a guinea-pig. The animal was injected with guinea-pig-rabbit hæmolytic serum, prepared by injecting the red blood corpuscles of the guinea-pig into the rabbit. Before its injection the serum was mixed with stomach cells for one hour. The stomach cells have failed to remove the hæmolysin from the serum.

# *The Influence of Increased Barometric Pressure on Man.—No. I.*

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(Received January 16,—Read February 15, 1906.)

## *Introduction.*

The classical researches of Paul Bert, (1) confirmed in recent years by v. Schrötter (2) and his co-workers, and also by Leonard Hill and J. J. R. Macleod (3 and 4), have demonstrated beyond question that the ill results observed in caisson workers and divers are to be attributed entirely to injudicious rapidity of decompression.

Experiments on animals have shown that every 100 c.c. of blood or tissue fluid dissolve, at body temperature, about 1 c.c. of nitrogen under one atmosphere of air; 2 c.c. under two atmospheres; 3 c.c. under three atmospheres, and so on (Hill and Macleod, Hill and Ham). (5)*

This nitrogen is set free as bubbles in the capillaries and tissue spaces when the decompression period is made too short, and by the embolism of some vessel, may produce symptoms varying in kind and severity.

One of us (L. H.) having determined, by numerous experiments on animals, that no ill effects follow exposure to pressures up to + seven atmospheres, if 20 minutes be allowed to each atmosphere for decompression, we determined to investigate the effects of high pressures of air upon ourselves.

The records of caisson works and the operations of deep sea divers show that owing to the rapid rates of decompression at present employed by engineers and divers, very great risk is incurred by workers in caissons at pressures of + 3 atmospheres, and by divers at depths of from 100 to 150 feet. As, however, divers usually stop a very brief time, while caisson workers outstay a shift of from 2 to 4 hours, the body fluids of the latter become saturated with nitrogen, hence their greater danger at lower pressures.

The limit for practical diving work is fixed by the great increase of mortality and illness which occurs at depths much exceeding 100 feet, while at less depths than this, accidents are by no means infrequent; being occasionally very severe or fatal in character.

The Admiralty set 120 feet as the limit of work for their divers, while the most daring pearl and sponge fishers sometimes reach depths of 145 feet; in this latter group accidents are numerous. Lambert, the famous diver employed by Messrs. Siebe and Gorman, salved £100,000 at a depth of about 160 feet. On each descent he passed about 20 minutes below, and about the same time in ascending. On the last journey he stayed longer and became affected on his return to the surface, permanently losing the power to retain his urine. Lambert was the man who stopped the flooding of the Severn Tunnel, going through the tunnel (dark and full of water) in a Fleuss dress to a distance of a quarter of a mile from the shaft, and closing the flood gates, which had been left open; his courage deserves to be recalled. Another diver, Erostabe, salved treasure from a depth of 171 feet, and yet another, Ridyard, from 160 feet. These three divers of Messrs. Siebe and Gorman hold the record for successful work carried out at great depths. Two other divers of the same firm, in order to test a patent kind of diving apparatus, descended to 189 and 192 feet respectively. One of these divers (Walker) tells us he was about 50 minutes over the job, taking 30 minutes to ascend. He ascribes his immunity from accident throughout his career as a deep diver to his habit of slow ascent. The deepest dive on record is one of 204 feet (+88½ lbs. pressure); the diver who made this record died from the effects of too rapidly mounting to the surface.

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* Bohr ('Nagel's Handb. d. Physiologie,' 1905, vol. 1, p. 117) gives the coefficient of absorption of arterial blood exposed to an atmosphere of $N_2$, at body temperature as 1·26.

In 1894, at Bordeaux, H. Hersent, (7) an engineer in charge of caisson works, having first experimented on animals, found three workmen willing to submit themselves to high pressures of air. These men were enclosed in a steel chamber, and the experiments were conducted under the observation of a commission composed of five members of the Bordeaux Faculty of Medicine. Two of the workmen had had previous experience of compressed air.

In one experiment the subject was compressed to +4·800 kilos. per square centimetre (+68·27 lbs. per square inch) in 35 minutes, remained under this pressure 1 hour, and was decompressed in 2 hours 3 minutes. On quitting the chamber the man experienced a few "picotements," which lasted for half-an-hour, but no other unfavourable symptoms. In a second experiment, a pressure of +5·000 kilos. (+71·16 lbs. per square inch) was attained, without any subsequent ill effects beyond a few "picotements."

Finally, the same subject was compressed to +5·400 kilos. (+76·81 lbs. per square inch) in 45 minutes, remained under the pressure 1 hour, and was decompressed in 2 hours 25 minutes. The effects are recorded in these words: "A ressenti peu de picotements, cela tient aux bains sulfureux pris les jours précédents." (8).

Hersent's experiments justify his conclusion that "avec quelques précautions en sus de celles qu'on prend ordinairement, les hommes peuvent être comprimés et décomprimés sans danger pour leur vie, et que même leur santé n'est pas menacée quand on atteint des pressions allant jusqu'à 5 kg. 400." (9)

Hersent and his medical colleagues do not appear to have entered the pressure chamber themselves, so that we are not in possession of an accurate record of the subjective effects as noted by trained scientific observers. One of our objects therefore has been to study in detail the subjective and physiological changes induced by greatly increased barometric pressures; another object has been the investigation of the respiratory exchange under the same conditions. In the present memoir we shall communicate the results already obtained.

## PART I.

Our experiments have been carried out in a steel cylinder kindly placed at our disposal by Messrs. Siebe and Gorman, the eminent firm of naval engineers, to whom we are further indebted for much valuable assistance.

This cylinder (*vide* photograph, p. 446) had a capacity of 42·2 cubic feet, and was provided with a mattress, blanket, and pillows, enabling the subject to adopt a comfortable attitude. Compression was effected by means of a two-cylinder motor-driven pump, which could raise the pressure to +6 atmospheres in about 40 minutes. Two decompression taps were provided, with fine bores, permitting very careful adjustment of the rate of escape. The chamber was also fitted with electric light, bell, telephone, and a thick glass observation window; the latter, however, was subsequently covered with a steel shutter for greater security. The pressure was measured by a Bourdon spring gauge, which had been tested for correctness. We shall now give an account of a typical experiment. The description is reproduced from notes taken at the time:—

### *Experiment II.* 29.11.05.

The subject* (M. G.) entered the chamber at 10.40 A.M. In order to avoid any accumulation of $CO_2$, a constant ventilation at the rate of 25 litres per minute was maintained.

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* The measurements, etc., etc., of the two subjects were: L. H., age 39, weight (in clothes) 87½ kilogrammes, height 1·81 metres, vital capacity 3500 c.c., tidal air 510 c.c.; M. G., age 25, weight 53 kilogrammes, height 1·65 metres, vital capacity 4000 c.c., tidal air 300 c.c. Both were in good physical condition.

<table>
<tr><th>Time.</th><th>Temperature of chamber.</th><th>Pressure.</th><th>Notes.</th></tr>
<tr><td>10.40 A.M.</td><td>57° F.</td><td>+0</td><td></td></tr>
<tr><td>10.50</td><td>62</td><td>—</td><td></td></tr>
<tr><td>10.55</td><td>—</td><td>+16 lbs.</td><td>Voice becoming nasal and metallic.</td></tr>
<tr><td>11.5</td><td>67</td><td>—</td><td></td></tr>
<tr><td>11.20</td><td>69</td><td>+62 lbs.</td><td>Sensation of slight vertigo.</td></tr>
<tr><td>11.34</td><td>68[*]</td><td>+92 „ [†]</td><td></td></tr>
<tr><td colspan="4">Between 11.25 and 11.40 articulation was difficult, and the subject experienced some trouble in making himself heard through the telephone.</td></tr>
<tr><td>11.55</td><td>—</td><td>+77 lbs.</td><td></td></tr>
<tr><td>12 noon</td><td>65</td><td>—</td><td></td></tr>
<tr><td colspan="4">Subject quite comfortable, voice still nasal but easier to produce and much more audible.</td></tr>
<tr><td>12.4 P.M.</td><td>—</td><td>+72 lbs.</td><td></td></tr>
<tr><td>12.10</td><td>—</td><td>—</td><td>Pulse, 40. Respirations, 9 per min.</td></tr>
<tr><td>12.37</td><td>64</td><td>+52 lbs.</td><td></td></tr>
<tr><td>1.0</td><td>63</td><td>+31 „</td><td>Voice much better.</td></tr>
<tr><td>1.20</td><td>63½</td><td>—</td><td>Pulse, 42.</td></tr>
<tr><td>1.51</td><td>—</td><td>+0</td><td></td></tr>
</table>

Period of compression, 54 minutes.

Period of decompression, 2 hours 17 minutes.

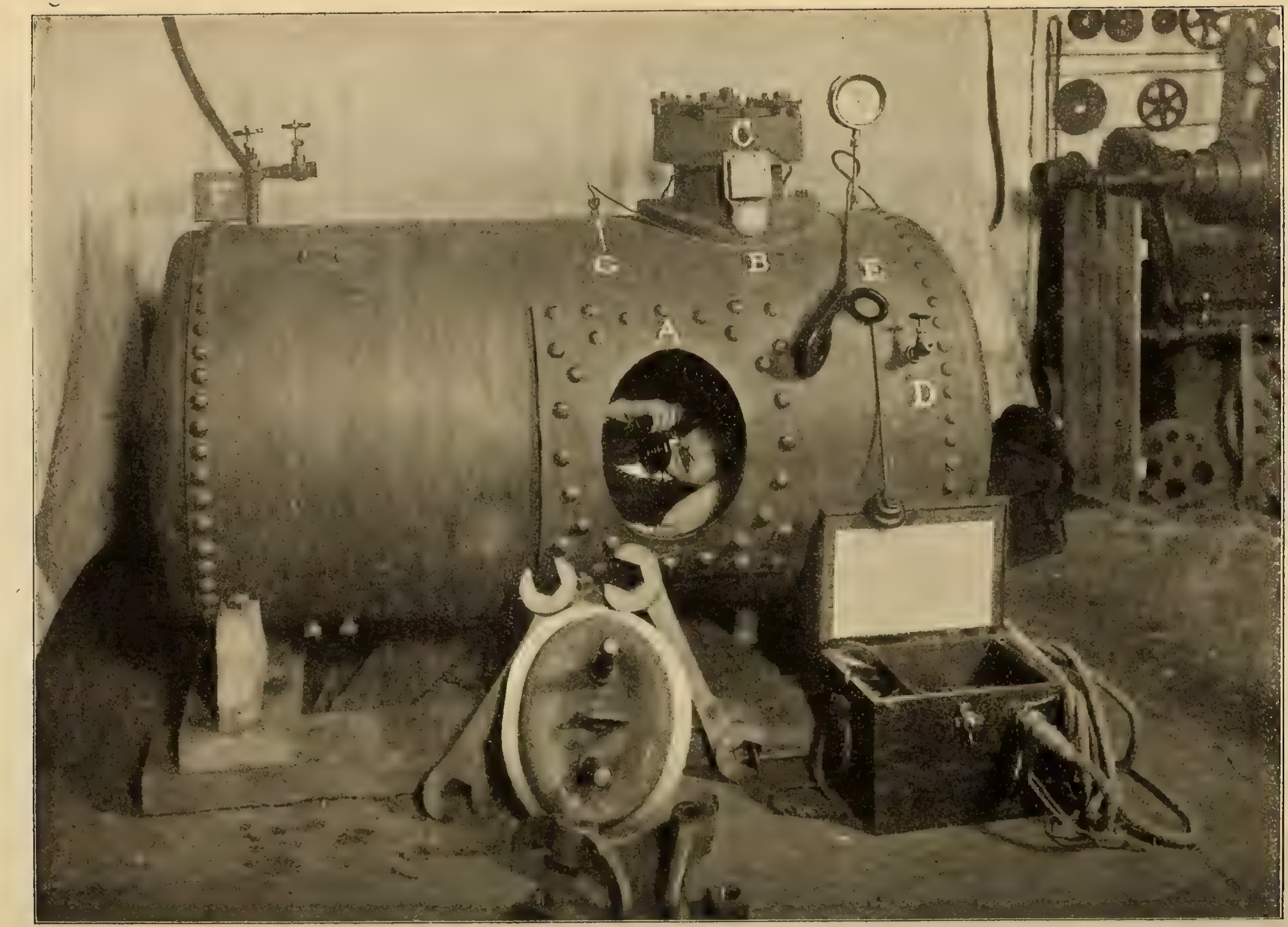
On quitting the chamber some itching was perceived in both forearms, especially the right. In about 20 minutes neuralgic pains were felt, localised in the radial side of the left forearm. These pains gradually increased in intensity, spreading up the arm; then, after remaining moderately intense for five minutes, they gradually subsided. Several minutes later (about one hour after leaving the chamber) similar pain was experienced in the right forearm. This however did not spread upwards, was less severe and quickly subsided. An hour and a half after leaving the cylinder the subject felt quite well and no subsequent ill effects resulted. As will appear later, there is good reason to suppose that the slight discomfort present at the conclusion of this experiment is attributable to the fact that the subject remained almost completely at rest during decompression. We may therefore conclude that an adult may be safely submitted to a total barometric pressure of at least 7 atmospheres, which is, we believe, a limit higher than any previously reached.

In the course of our investigation the following pressures have been attained :—

| Subject, L. H. | Subject, M. G. |
|---|---|
| 75 lbs., once. | 90 lbs., once. |
| 60 „ twice. | 75 „ three times. |
| 45 „ „ | 60 „ four „ |
| 30 „ four times. | 45 „ five „ |
| | 30 „ seven „ |

[*] Wet cloths were placed on the cylinder at this time.

[†] This reading was verified by Mr. J. A. Craw, who was present during the whole course of the experiment.



VIEW OF CYLINDER (OPEN), WITH WORKMAN INSIDE.

A. Manhole. B. Electric bell. C. Observation window (closed). D. Decompression tap.
E. Telephone. F. Compression pipe. G. Electric light wire.

In no case have any severe after effects resulted. The maximum pressure in our series corresponds to a water depth of 210 feet, which is 90 feet beyond the limit fixed by the Admiralty for their divers.

Supposing the special diving bell designed by one of us (L. H.) for the slow decompression of divers were employed, it seems quite possible that work might be carried out safely at a depth of 210 feet. Even a greater depth than this might be attained by an intrepid man, for the limit appears to be fixed by the pressure at which the toxic effects of high tension oxygen become an immediate danger.

These effects have been studied by Paul Bert, Lorrain Smith (10) and Hill and Macleod (3). When the partial pressure of oxygen reaches 2 atmospheres (corresponding to 10 atmospheres of air, or a depth of about 350 feet of water) convulsions may occur in animals within 20 minutes. The limit of *possible* safe working is therefore about 250 feet. Conceivably this limit might be extended by diluting the air with nitrogen so as to lower the partial pressure of the oxygen, but we do not claim more than that our experiments show the safe diving depth may be increased up to 210 feet.

The responsibility of those who allow short decompression periods in caisson works is clear; every death or case of paralysis from air embolism must be set down to the negligence of the contractor.

Next, as to the sensations we felt under pressure: the feeling of discomfort in the ears and deafness, due to a difference in air pressure within and without the tympanum, is too well known to need description. Owing probably to a catarrhal condition, we were unable to open our Eustachian tubes by merely swallowing, and were compelled to resort to a forced expiratory effort with mouth and nose shut, the latter being held tight by the finger and thumb.

To one of us (L. H.) who had not practised beforehand the opening of his Eustachian tubes, the first *séance* was most disturbing. The sensation of increasing deafness and discomfort, more than discomfort, in the ears, with no obvious cause, and the inability to gain relief by the recognised method of swallowing, produced a feeling of mental distress which led to his signalling to terminate the experiment. Once having learned the method of opening his tubes, no such trouble resulted on subsequent occasions.

As to whether one possesses any real sense of the amount of pressure, the answer must be in the negative. V. Schrötter and his co-workers (11), who made observations in caissons sunk in the Danube at from +0·5 to +2·65 atmospheres, say that: "Bleibt nun der Druck stationär, verweilt die Person auf längere Zeit unter einem bestimmten Drucke, so hört mehr oder minder rasch, oft mit einem Schlage, jegliche unangenehme Sensation im

Ohre auf, nur das Gefühl von Dumpfheit, das Gefühl eines vermehrten Widerstandes im Ohre, wird in der Mehrzahl der Fälle, besonders von Ungewohnten, wahrgenommen."

We found that all distinct sensations of pressure in the ears were relieved immediately the pump ceased its strokes, and the pressure in the chamber became constant. Our hearing was as acute and, in the opinion of L. H., more acute than normally. The signal of a tap with an iron spanner on the outside of the chamber was, to L. H., painful in its intensity.

Apart from the feelings of nervousness at being exposed to so high a pressure (which at times were somewhat acute, especially when we were not engaged in analytical work), we could not detect any real sense of pressure, and certainly noticed no abnormality in our bodily functions, with the trifling exception of the voice. Thus during Experiment XV the subject (L. H.) when at +60 lbs., wrote: "Very nervous all through experiment; whenever time for thought, the feelings of pressure, if any, due to non-equilibration of ears when pressure is rising." During the same experiment, when the subject learnt he was at +55 lbs., he wrote: "Thought one was lower until told. No real sense of pressure except lip and voice change." In another experiment, M. G. was nearly two atmospheres too low in his estimate of the pressure, while in a third experiment made at a period when custom had lessened the nervous effect, he replied to a question at +60 lbs., "no sense of pressure."

The voice changes, observed in all caisson workers, were well marked in ourselves. The alteration is distinct at +1 atmosphere, and very marked at +3 atmospheres. The voice has a peculiar nasal and metallic quality, losing the individual characteristics of the speaker. Thus to L. H., when speaking in the chamber, under pressure, his voice appeared like that of M. G. under pressure. So close was the resemblance that L. G. could fancy himself outside and listening to M. G. through the telephone.

At +3 atmospheres the power to whisper or whistle is almost entirely lost. L. H., who retained the power somewhat longer than M. G., could just make an audible whistling note at this pressure.

This loss of the fine vibratile movements of the tongue and lips, a loss probably resulting from the damping effects of the dense air, leads to a false sense of anæsthesia in the former parts. This conception of anæsthesia is interesting, as being solely excited by a lack of normal movement.

V. Schrötter and others have laid stress on the diminished frequency of the pulse and lowered blood pressure of caisson workers. Our observations are not sufficiently extensive to permit of any final pronouncement; but, so far as they go, we are unable to detect any *definite* change in the pulse frequency. For instance, in Experiment II, M. G.'s pulse was at the rate of 40 per minute at +70 lbs., and 42 at +63 lbs. In Experiment XIV, it was 41 per minute, at +50 lbs., 30 at +30 lbs., 42 at +10 lbs., and 41 at +2·3 lbs. This subject's pulse is normally slow, being rarely above 60 per minute in the sitting posture; hence although there appears to have been a diminution in frequency, the change is not nearly so striking as in the cases tabulated by V. Schrötter (12).* L. H. found no alteration in his pulse-rate at +5 atmospheres.

Our observations on the blood pressure have not been at all complete. The Hill and Barnard pocket sphygmometer, depending as it does upon a column of air acting as an elastic spring, is not a satisfactory instrument for high pressure work, the viscosity of the dense air lessening the excursion of the pulse very greatly.

We came to the conclusion that it was an important matter during the decompression to move in turn every muscle and joint of the body, and to change one's position frequently, so as to keep the capillary circulation active in every part. In the brain, spinal cord, and abdominal organs this circulation is kept active by the work of the respiratory pump. In the limbs, muscles, fat of the back and chest, on the other hand, the movement of the blood and lymph back to the heart depends mostly on changes of posture and the expressive action of contracting muscles. The following observations support these views.

In Experiment XIII M. G. was decompressed from +75 lbs. in 95 minutes. During decompression he flexed and extended all the limb joints at frequent intervals, with the exception of the knees. Subsequently pain and stiffness were detected in the knees and nowhere else.

In Experiment XIV the same subject was decompressed from +5 atmospheres in 120 minutes. During the compression all the limb joints, including the knees, were repeatedly moved. No after effects of any kind were experienced. A further difference between the two experiments was that in the second a pause of about five minutes was made at each atmosphere for analytical purposes. As in each of the experiments followed by pain (in the case of M. G.) no such pauses occurred, it is possible, but we think not probable, that these may also play a part in hindering the development of after effects.

The most interesting experiment in this connection is No. XV. L. H. was decompressed from +5 atmospheres in 105 minutes, a pause of five minutes being made at each atmosphere. During the decompression movements of the joints and muscle of the limbs and back were carried out regularly. On emerging from the cylinder, beyond a few "picotements," no unpleasant symptoms were noticed.

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* In Schrötter's cases there was no direct relation between barometric pressure and pulse frequency.

On the next day the subject wrote as follows: "The only place I did not move and massage was the front of the chest, where I have plenty of subcutaneous fat. In the evening painful places were felt in the subcutaneous tissues of the anterior thoracic region; one spot under each nipple, one across the right side of the chest about the level of the ensiform cartilage, another above the left axilla in front, and one over the right upper arm in front. A red or purplish rash appeared over these tender places. They felt like a spot in which a subcutaneous injection of water has been made. Next morning the tenderness was better but still evident, and the rash was subsiding."

Forty-eight hours after the experiment this purpuric rash was still discernible, and was shown to Dr. W. Bulloch and other pathologists. An eruption occurred in a very severe case of caisson illness seen by Heller Mager and v. Schrötter (13). They give a plate of the eruption, which is described in these terms: "Haut der linken Schulter und des linken Armes an der Aussenseite, besonders in der Gegend des Olekranon und des äusseren Condylus sowie in der Gegend des Biceps mit lividen, bläulichrothen netzförmig verzweigten, inselförmigen Flecken bedeckt, ebensolche auch am Handrücken." The arm of this sufferer was much swollen and intensely painful. These observations then show the extreme importance of active movement and massage during decompression; instructions should be given to all caisson workers to perform such movements while in the air lock.

We believe the tenderness and the rash were caused by small bubbles embolising the vessels of the subcutaneous fat in the case of L. H. The pair felt by M. G. was probably due to small bubbles in the nerve sheaths in the first case, in the knee joint in the second.

## PART II.

The next stage of our investigation was devoted to an inquiry as to the changes in the percentage of alveolar $CO_2$ under the altered conditions.

We have employed the method described by Haldane and Priestley (14) The subject breathes through a wide-bored rubber tube; after a normal expiration he expires deeply and then closes the end of the tube with his tongue. A sample was taken from the wide tube into Haldane's portable $CO_2$ analyser, and examined. A bench fitted up in our cylinder enabled the subject to collect and examine samples with ease. It may be remarked that it is necessary to replace the corks at the bottom of the water bath in Haldane's apparatus by well-fitting rubber ones, as the air is compressed in the corks, which leak at high pressures. Owing to the loss of the water jacket some of our earlier experiments were unsuccessful. Great care is also necessary in readjusting the potash levelling tube, as when the chamber is closed a slight fall of pressure is almost inevitable owing to escape round the washer of the door.

Haldane and Priestley have shown that the respiration is so regulated as to maintain a constant tension of $CO_2$ in the alveolar air, which is generally about 5 per cent. of an atmosphere. Now supposing the metabolism to be unaffected by changes of pressure, and the regulation of respiration to continue the same, the amount of $CO_2$ in the alveolar air must vary inversely as the pressure attained.

Thus if $p$ be the percentage of $CO_2$ at normal pressure, then we should have, at two atmospheres, $p/3$ per cent. of $CO_2$ in the alveolar air. It will be seen that these conditions were almost exactly realised by us. The following table gives the result of two typical experiments. The figures in brackets give the percentages reduced to + 0 lb. in accordance with the above principle. Strictly speaking, the exact height of the barometer should have been recorded, and another correction ought to have been made for charges of temperature in the cylinder. As there is however a necessarily large experimental error, we think it needless to allow for these minor differences, and have accordingly assumed the normal atmospheric pressure to be 15 lbs. to the square inch, and neglected the temperature :—

*Experiment XIV.* 10.1.06. *Subject, M. G.*

| Percentage of CO in alveolar air. | | Pressure. |
|---|---|---|
| 5·3 (5·3) | | + 0 lbs. |
| 0·9 (5·4) | (Mean of two) | +75 „ |
| 1·0 (5·0) | | +60 „ |
| ? 1·3 (5·0) | | +45 „ |
| 1·8 (5·4) | | +30 „ |
| 2·7 (5·4) | | +15 „ |
| 5·4 (5·4) | | + 0 „ |

*Experiment XV.* 10.1.06. *Subject, L. H.*

| Percentage of $CO_2$ in alveolar air. | Pressure. |
|---|---|
| 4·7 (4·7) | + 0 lbs. |
| 0·9 (4·5) | +60 „ |
| 0·7 and 0·8 (4·5) | +75 „ |
| 0·95 (4·75) | +60 „ |
| 1·2 (4·8) | +45 „ |
| 1·8 (5·4) | +30 „ |
| 2·5 (5·0) | +15 „ |
| 5·0 (5·0) | + 0 „ |

The next tables comprise all our results. The figures vertically beneath one another refer to the same experiment :—

Subject, M. G.

| Pressure. | Alveolar percentages of $CO_2$. | | | | | |
|---|---|---|---|---|---|---|
| lbs. | | | | | | |
| 0 | 5·3, 5·4 | 5·3 | 5·5 | 5·7 | 5·5, 5·7 | 5·3, 5·4 |
| 8 | 3·3 (5·06)<br>mean | — | — | — | — | — |
| 15 | 2·3 (4·6)<br>mean | — | — | — | — | 2·7 (5·4) |
| 16 | — | — | — | 2·7 (5·58) | 2·7 (5·58) | — |
| 22 | — | — | 2·1 (5·18) | — | — | — |
| 30 | — | — | 1·8 (5·4) | — | — | 1·8 (5·4) |
| 31 | — | — | — | 1·8 (5·52) | 1·8 (5·52) | — |
| 45 | — | — | — | — | — | ?1·3 (5·2) |
| 46 | — | — | — | — | ?1·3 (5·3) | — |
| 60 | — | — | — | — | — | 1·0 (5·0) |
| 61 | — | — | — | — | 0·9 (4·6) | — |
| 75 | — | — | — | — | — | 0·9 (5·4) |

Subject, L. H.

| Positive pressure. | Alveolar percentages of CO. | | | | |
|---|---|---|---|---|---|
| lbs. | | | | | |
| 0 | 5·2, 5·3, 4·9 | 4·9, 5·0 | 5·15 | 4·9, 5·0 | 4·7, 5·0 |
| 4 | 3·5, 4·0 (4·8) | — | — | — | — |
| 9 | — | 3·35 (5·35) | — | — | — |
| 18 | — | 2·5 (5·48) | — | — | — |
| 23 | — | 2·1 (5·3) | — | — | — |
| 17 | — | — | 2·5 (5·3) | — | — |
| 31 | — | 1·85 (5·7) | 1·7 (5·2) | — | — |
| 14½ | — | — | — | 2·5 (4·9) | — |
| 30 | — | — | — | 1·65, 1·7 (5·0) | 1·8 (5·4) |
| 44½ | — | — | — | 1·25, 1·3 (5·1) | — |
| 45 | — | — | — | 1·3 (5·2) | 1·2 (4·8) |
| 52 | — | — | — | 1·1 (4·9) | — |
| 60 | — | — | — | 0·95, 1·0 (4·88) | 0·9, 0·9 (4·5) |
| 75 | — | — | — | — | 0·7, 0·8 (4·5) |
| 15 | — | — | — | — | 2·5 (5·0) |

We think these results show so close an agreement with the theoretical values as to support the conclusion that changes in the percentage of carbon dioxide in the alveolar air depend solely upon the physical conditions. No increase or decrease in the pulmonary output of $CO_2$ occurs. Metabolism, then, in so far as it can be determined by an investigation of the alveolar air, is not affected by increasing the barometric pressure. It is scarcely necessary to add that this criterion is by no means adequate to sustain the *final* conclusion that metabolism is, in fact, unaltered by the atmospheric conditions; so far as it goes, however, it is in favour of such an inference. Summing up the results of the present investigation :—

It is proved that—

(1) A man can be submitted to a total pressure of seven atmospheres without untoward effects, provided decompression be affected gradually, and the capillary circulation be aided by repeated contractions of muscles, joint movements, and charges of posture.

(2) We have no sense of increased barometric pressure so long as the former is constant.

It is probable—

(1) That the subjective effects of increased pressure, apart from voice changes and lip anæsthesia, depend upon psychical conditions such as anxiety and excitement.

(2) The changes in the percentage of carbon dioxide in the alveolar air are conditioned solely by physical variations, and not by any increase or diminution in the respiratory metabolism.

In conclusion we would remark that we are unable to find any evidence in support of Snell's (15) opinion, that the presence of $CO_2$ in the respired air exercises a peculiarly unfavourable influence under increased pressure. Thus in one experiment the percentage of $CO_2$ in the chamber air, at + 31 lbs. was 0·62 (equivalent to over 1·8 per cent. at + 0), and no untoward results occurred on decompression.

These researches were carried out with the aid of a grant from the Royal Society Government Grant.

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# *Studies on Enzyme Action—Lipase.*

By Dr. Maurice Nicloux.

(Communicated by Professor W. D. Halliburton, F.R.S. Received January 16,—Read February 1, 1906.)

In a recent number of the 'Proceedings of the Royal Society'* Dr. Henry G. Armstrong published a paper with the above title. I beg leave to draw attention to the work† I have performed on the saponification of fats by castor-oil seeds, and, without entering into detail, to state my general conclusions. These are as follows:—

(*a*) By mechanical means it is possible to separate the cytoplasm of the castor-oil seeds from all the other cellular elements, particularly from the aleurone grains.

(*b*) Pure cytoplasm prepared as above alone has the property of hydrolising fats; its power is considerable.

(*c*) It acts on the fats in the same way as an enzyme, and follows all the laws of enzyme action.

(*d*) Nevertheless the active substance of which cytoplasm is but probably the support is not an enzyme; this substance, which I proposed to call "lipaseïdine," is destroyed by water as soon as it is no longer protected by fats.

(*e*) It is possible to repeat *in vitro* with isolated cytoplasm hydrolysis of the fatty matter such as occurs in the seed at the time of germination.

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* 'Roy. Soc. Proc.,' B, vol. 76, p. 606.

† These were published in a series of notes in the 'Comptes Rendus de l'Academie des Sciences':—"Sur un procédé d'isolement des substances cytoplasmiques," 'Compt. Rend.,' 1904, vol. 138, p. 1112; "Sur le pouvoir saponifiant de la graine de ricin," 'Compt. Rend.,' 1904, vol. 138, p. 1175; "Étude de l'action lipolytique du cytoplasma de la graine de ricin," 'Compt. Rend.,' 1904, vol. 138, p. 1288; "La propriété lipolytique du cytoplasma de la graine de ricin n'est pas due à un ferment soluble," 'Compt. Rend.,' 1904, vol. 138, p. 1352; "Mécanisme d'action du cytoplasma (lipaseïdine) dans la graine en voie de germination; realisation synthétique de ce mécanisme," 'Compt. Rend.,' 1904, vol. 139, p. 143; and later in a general memoir, "La saponification des corps gras," 'Revue Générale des Sciences,' 16ème Année, No. 23, 15 Decembre, 1905, pp. 1029—1037.

# *On the Function of Silica in the Nutrition of Cereals.—Part I.*

By A. D. Hall and C. G. T. Morison.

(Communicated by Professor H. E. Armstrong, F.R.S. Received December 22, 1905,—Read February 1, 1906.)

(From the Lawes Agricultural Trust Committee.)

## 1. *Introduction.*

The presence of silica in plants was first demonstrated by the analyses of De Saussure,[*] who pointed out that the Gramineæ were particularly distinguished by the large proportion of this constituent present in their ash. Liebig, who classified plants as "silica plants," "lime plants," and "potash plants" according to the predominance of one or other of these constituents in their ash, in accordance with his "mineral theory," regarded the silica as a necessary element in plant nutrition. This view led Way[†] to introduce as a cereal manure a rocky material derived from the Upper Greensand near Farnham, which contained a considerable proportion of silicate easily soluble in acids. But when Sachs[‡] succeeded in maturing maize plants in water cultures containing no silica, whereby the proportion of silica in the ash of the mature plant was reduced from the normal 20 per cent. or so to as little as 0·7 per cent., it became evident that silica could no longer be placed in the same category as phosphoric acid and potash as essential elements of plant nutrition, and Jodin[§] raised four successive generations of maize in water cultures without any supply of silica beyond that contained in the original seed.

Other investigators again showed that the stiffness of cereal straw, which had been attributed to the presence of silica, depends on the development of the internodes under the influence of such factors as illumination and exposure.

Henceforward little or no importance seems to have been attached to the presence of silica, yet, as the following ash analyses show, it forms a constant and considerable proportion in the ashes of certain plants, though it is almost absent from the majority.

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[*] 'Recherches sur la Végétation,' Paris, 1804.

[†] 'Roy. Agric. Soc. Journ.,' vol. 14, 1853, p. 225.

[‡] 'Flora,' 1862, p. 52.

[§] 'Ann. Agron.,' vol. 9, 1883, p. 385.

Table I.—Percentage of Silica in Ash.

| | $SiO_2$, per cent. | | $SiO_2$, per cent. |
|---|---|---|---|
| Wheat straw (Rothamsted mean)... | 62·1 | Hops, leaves (Wolff, mean)............ | 21·1 |
| „ grain „ ... | 0·7 | „ cones „ ............ | 17·2 |
| Barley straw „ ... | 46·0 | Beech leaves „ ............ | 31·0 |
| „ grain „ ... | 18·3 | Larch needles „ ............ | 22·5 |
| Oat straw (Wolff, mean)... | 46·1 | *Calamus Rotang* (Wolff, 1 anal.)... | 68·0 |
| „ grain „ ... | 36·3 | *Bambusa arundinacea* „ ... | 28·3 |
| Rye grass (*Lolium perenne*) „ ... | 26·7 | *Sphagnum palustre* „ ... | 61·8 |
| Maize (whole plant) „ ... | 43·0 | *Pteris aquilina* „ ... | 43·7 |
| Sugar cane „ ... | 56·4 | *Equisetum arvense* „ ... | 41·7 |
| | | *Erica Tetralix* „ ... | 48·4 |

Owing to the inevitable presence of external dust and dirt upon plant material before analysis, it is almost impossible to say whether the small amounts of silica found in the ashes of many other plants are accidental or inherent.

But while it has been demonstrated that silica is not essential to the nutrition even of the cereals, it is hardly likely that a material present to the extent of 60 per cent. of the mineral constituents, as in the ash of wheat-straw, can be wholly without use in the economy of the plant. The only experiments, however, which throw any light on its function appear to be those of Wolff and Kreutzhage.* These investigators grew oats in culture solutions of the type usually described as complete, but further divided into three series, receiving soluble silica in considerable quantity, in a small quantity, and not at all. They observed that while the total growth was not much increased by the presence of silica, the proportion of grain formed was considerably raised, a precisely similar effect to that brought about by an addition of phosphoric acid to culture solutions deficient in that element. Hence they concluded that the action of silica and of phosphoric acid were in some way related, the former acting, however, indirectly on grain formation by promoting the migration of the food materials.

With this exception the possibility that silica plays any part in plant nutrition appears to have been ignored, just as its practical use in the manuring of cereals has been discontinued. Observation, however, of some of the plots at the Rothamsted Experimental Station, which have long been subjected to a manuring with soluble silicates, seemed to show that the question of the function of silica required further consideration, and an examination of the records indicated at once that the appearances noticed were not accidental, but had persisted from year to year.

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* 'Land. Versuchsstationen,' vol. 30, 1884, p. 161.

### 2. *Field Experiments at Rothamsted with Soluble Silicates.*

At Rothamsted sodium silicate has been applied as a manure to certain of the experimental plots over long periods of time, and shows regular and well-marked effects.

On the permanent grass plots in the Park, which is cut for hay every year, there are two plots receiving similar heavy applications of ammonium salts, phosphates, and potassium, sodium, and magnesium sulphates. One of these, which receives sodium silicate also, yields a crop exceeding by about 10 per cent. the crop of the parallel plot without sodium silicate, taking an average over the last 42 years. It is possible, however, that the weakly-held sodium base has some part in this action, by neutralising the acidity produced in the soil by the continued use of ammonium salts. This difficulty of interpretation does not, however, apply to the barley plots.

In Hoos field, on which barley has grown every year since 1852, one series of plots receives sodium nitrate with various combinations of mineral manures, so as to provide plots receiving (1) nitrogen alone; (2) nitrogen and phosphoric acid without potash; (3) nitrogen and potash without phosphoric acid; and (4) a complete manure.

Since 1864 one-half of each of these plots has been cross-dressed with sodium silicate; hence the effect of the silicate is seen in conjunction with each of the elements of a complete manure. The average results obtained are set out in Table II.

Table II.

| Plot. | Manures per acre. | | | | | Average over 41 years, 1864—1904. | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Grain. | | Straw. | |
| | Sodium nitrate. | Superphosphate. | Potassium sulphate. | Sodium sulphate. | Magnesium sulphate. | Without silicate. | With silicate. | Without silicate. | With silicate. |
| | lb. | cwt. | lb. | lb. | lb. | Bushels. | Bushels. | cwt. | cwt. |
| 1 | 275 | — | — | — | — | 27·3 | 33·8 | 16·2 | 19·8 |
| 2 | 275 | 3·5 | — | — | — | 42·2 | 43·5 | 24·6 | 25·8 |
| 3 | 275 | — | 200 | 100 | 100 | 28·6 | 36·4 | 17·9 | 21·7 |
| 4 | 275 | 3·5 | 200 | 100 | 100 | 41·2 | 44·5 | 25·3 | 27·6 |

In this case only a normal amount of nitrogen is supplied in the form of sodium nitrate, a neutral salt, so that there is no acid to be neutralised by the soda of the sodium silicate. The beneficial effect of the sodium silicate is chiefly shown on Plots 1 and 3, and there is little gained by its use on Plots 2 and 4. Now Plot 3 is abundantly supplied with alkaline salts in the shape of sodium nitrate and sulphates of sodium, potassium, and magnesium, so the addition of a further supply of sodium in sodium silicate would not be likely to produce any effect. Rather, if the sodium were the active constituent, would its effect be seen on Plot 2, which receives no alkaline salts beyond the sodium in the sodium nitrate common to all the plots. The notable fact is that the effect of the sodium silicate is seen only on the two Plots 1 and 3 suffering from phosphoric acid starvation, because they have been cropped for so many years without the application of any phosphates. The silica, in fact, would seem to partially replace or to do the work of the superphosphate supplied to Plots 2 and 4.

Such an opinion, derived from the yield, may be confirmed by an examination of the plots when approaching ripeness. The most striking feature at that time is the deferred maturity of the barley on the plots without phosphoric acid; they remain of a greener colour, and are still erect at a time when the barley on the normal plots has turned down and begun to yellow for harvest. This ripening effect of phosphoric acid finds a parallel, though to a smaller degree, on the half plots receiving sodium silicate. On Plots 1 and 3, which are without phosphoric acid, the portions receiving sodium silicate are always riper by a few days than the other halves which get neither phosphoric acid nor silica.

A series of analyses of the ash of the barley grown on these plots in 1903, a wet and sunless year, and 1904, a normally warm season, also serve to strengthen the idea that the action of the silica is in some way bound up with that of the phosphoric acid in the plant. Table III shows the percentages of phosphoric acid and silica in both grain and straw on the four plots, each of which is subdivided so as to be with and without silica.

It will be seen that the lack of phosphoric acid in the manure applied to Plots 1 and 3 is reflected in the diminished proportion of phosphoric acid in the ash of the grain, and still more in the low percentage present in the ash of the straw. When sodium silicate is added to the plots without phosphoric acid the proportion of phosphoric acid in the grain ash rises, but simultaneously it falls in the straw ash.

On the plots receiving phosphoric acid the silicate does not always cause an increase in the percentage of phosphoric acid in the grain ash, though as before it generally diminishes that in the straw ash.

On all the plots the sodium silicate causes an increase of silica in the ash of the grain, and particularly in that of the straw, indicating that under the ordinary soil conditions the barley plant does not obtain all the soluble silica it would otherwise appropriate.

Table III.—Hoos Field Barley.

Nitrogen and Pure Ash per cent. in Dry Matter, and Phosphoric Acid and Silica in Pure Ash.

| | Nitrogen. | | Nitrogen and phosphate. | | Nitrogen and potash. | | Nitrogen, potash, and phosphate. | |
|---|---|---|---|---|---|---|---|---|
| | Only. | With silica. | Only. | With silica. | Only. | With silica. | Only. | With silica. |
| | 1. | 1 S. | 2. | 2 S. | 3. | 3 S. | 4. | 4 S. |
| Grain. | | | | | | | | |
| 1903— | | | | | | | | |
| Nitrogen in dry matter... | 1·63 | 1·57 | 1·50 | 1·50 | 1·59 | 1·61 | 1·53 | 1·54 |
| Pure ash ,, ... | 1·74 | 1·98 | 2·27 | 2·37 | 1·78 | 1·96 | 2·36 | 2·36 |
| Phosphoric acid in pure ash | 35·80 | 37·74 | 42·27 | 42·64 | 35·54 | 36·11 | 41·83 | 44·31 |
| Silica in pure ash ......... | 14·19 | 18·67 | 16·43 | 20·60 | 15·81 | 18·00 | 16·95 | 19·71 |
| Ratio, $P_2O_5$ to N ......... | 0·38 | 0·48 | 0·64 | 0·67 | 0·40 | 0·44 | 0·64 | 0·68 |
| 1904— | | | | | | | | |
| Nitrogen in dry matter... | 1·79 | 1·72 | 1·52 | 1·46 | 1·58 | 1·73 | 1·46 | 1·45 |
| Pure ash ,, ... | 1·94 | 2·09 | 2·34 | 2·41 | 1·97 | 2·15 | 2·33 | 2·32 |
| Phosphoric acid in pure ash | 32·19 | 35·29 | 40·16 | 36·40 | 30·96 | 34·16 | 38·82 | 38·46 |
| Silica in pure ash ......... | 16·76 | 20·13 | 19·62 | 20·75 | 16·45 | 17·47 | 16·34 | 19·08 |
| Ratio, $P_2O_5$ to N ......... | 0·35 | 0·43 | 0·62 | 0·60 | 0·39 | 0·42 | 0·62 | 0·62 |
| Straw. | | | | | | | | |
| 1903— | | | | | | | | |
| Nitrogen in dry matter... | 0·53 | 0·43 | 0·43 | 0·41 | 0·56 | 0·50 | 0·42 | 0·44 |
| Pure ash ,, ... | 3·66 | 4·80 | 3·71 | 4·86 | 4·24 | 4·82 | 3·98 | 4·73 |
| Phosphoric acid in pure ash | 2·34 | 2·40 | 4·18 | 3·62 | 2·41 | 2·19 | 4·02 | 4·38 |
| Silica in pure ash ......... | 51·98 | 63·88 | 56·67 | 64·00 | 46·22 | 55·37 | 48·14 | 57·30 |
| Ratio, $P_2O_5$ to N ......... | 0·16 | 0·27 | 0·36 | 0·43 | 0·18 | 0·22 | 0·38 | 0·48 |
| 1904— | | | | | | | | |
| Nitrogen in dry matter... | 0·49 | 0·48 | 0·40 | 0·42 | 0·50 | 0·48 | 0·43 | 0·45 |
| Pure ash ,, ... | 4·07 | 5·00 | 4·36 | 5·09 | 4·61 | 5·29 | 4·19 | 5·01 |
| Phosphoric acid in pure ash | 2·66 | 2·13 | 4·47 | 4·17 | 2·48 | 2·02 | 4·78 | 3·96 |
| Silica in pure ash ......... | 44·00 | 52·54 | 47·19 | 51·28 | 35·91 | 44·07 | 37·43 | 44·13 |
| Ratio, $P_2O_5$ to N ......... | 0·21 | 0·23 | 0·49 | 0·51 | 0·23 | 0·22 | 0·47 | 0·44 |

As the application of a soluble silicate lowers the proportion of phosphoric acid in the straw while raising it in the grain, it would seem at first sight to act by facilitating the migration and utilisation in the grain of the initially small store of phosphoric acid derived from the soil.

But such an interpretation of the function of silica is not borne out if the whole amount of phosphoric acid removed by the crop from the soil on each plot be considered, instead of the proportion of phosphoric acid in the ash. It has already been shown that the use of sodium silicate on the no phosphoric acid plots, 1 and 3, results in a considerable increase of crop, and as the grain of this increased crop is somewhat richer and the straw only a trifle poorer in phosphoric acid than the grain and straw from the non-silicated portions of the plots, it follows that the whole crop manured with silica contains a greater total amount of phosphoric acid derived from the reserves of phosphoric acid in the soil. This extra phosphoric acid derived from the soil is itself sufficient to explain the greater yield brought about by the silicate without attributing to the silica within the plant any specific action in economising the phosphoric acid there present. If the function of the silica were to replace the phosphoric acid within the plant and enable it to be moved off to the active tissues and used over and over again, the larger crop due to manuring with silicates would not contain any greater amount of phosphoric acid, but the general growth of the plant, *e.g.*, the dry matter produced and the nitrogen assimilated, would be increased. Hence the ratio of the phosphoric acid to the dry matter and to the nitrogen would be lowered in proportion to the increased growth, conditions which are not realised in the cases under examination, where indeed the ratio of phosphoric acid to nitrogen is generally slightly raised by the applications of silicate.

The results on the other hand indicate that the silicate gives the plant such a stimulus as enables it to develop more vigorously and obtain more phosphoric acid from the soil, and that all the consequences observed follow from the increase of phosphoric acid thus brought about.

Wolff and Kreutzhage held that the function of the silica was to enable the plant to make fuller use of whatever phosphoric acid it had obtained from the soil, the Rothamsted results indicate that its action takes place earlier, in stimulating the plant to draw more efficiently upon the vast but dormant reserves of phosphoric acid in the soil.

### 3. *Effect of Silica on the Development of Barley in* 1904.

In order to study the question more closely it was decided in 1904 to trace the effect of phosphoric acid and silica upon the development of the barley on these plots at regular intervals from the time of flowering onwards. As the effect of phosphoric acid had been most evident in forwarding the maturation of the crop, it was considered that the later period of the growth of the crop need only be investigated, *i.e.*, the period during which the nutrition of the plant from the soil has largely ceased and assimilation is coming to a standstill, while the materials previously accumulated in the stem and leaf are migrating into the seed.

The method adopted was to take a certain number of rows of barley in the middle of each plot and remove the whole plant, as far as possible with the roots intact, for two yards up these rows, at weekly intervals from June 13 until harvest on August 8, or nine times in all. The plants were then air-dried after washing the roots free from soil, the grain when formed was separated from the straw, and both were finally dried in the steam oven, so as to obtain the weight of dry matter; although dealing with such small areas it is impossible to make more than a very approximate estimate of the yield per unit area. The dried material was ground, and after determinations of the nitrogen in one portion, the rest was burnt for ash, in which the pure ash, free from sand and charcoal, and the phosphoric acid and silica were determined.

The analytical results are set out in Appendix Tables VII to X, from which are derived the various curves of development now to be considered (figs. 1 to 11). Before however proceeding to a consideration of particular cases it will be convenient to trace by means of an average result for all the plots the general course of development in the later stages of the growth of the barley plant.

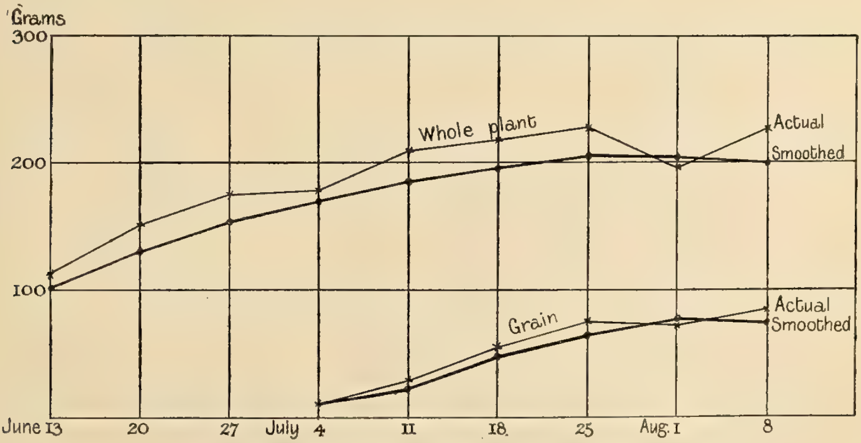

Fig. 1.—Dry Weights. Mean of all plots. Whole Plant and Grain.

Fig. 1 shows the mean dry weight of all the eight plots on each date, both of the whole plant and of the grain. The crop attained its maximum weight about July 18—25, after which it remained stationary and probably indeed declined slightly. The result shown for August 1 is clearly exceptional; on that date several of the plots happened to yield an exceptionally small number of stems on the area harvested.

For the better calculation of mean results the smoothed curve also shown in fig. 1 was drawn; by combining the smoothed dry weights read off this curve with the true mean percentages at each date were obtained the data contained in Table IV and expressed graphically in fig. 2.

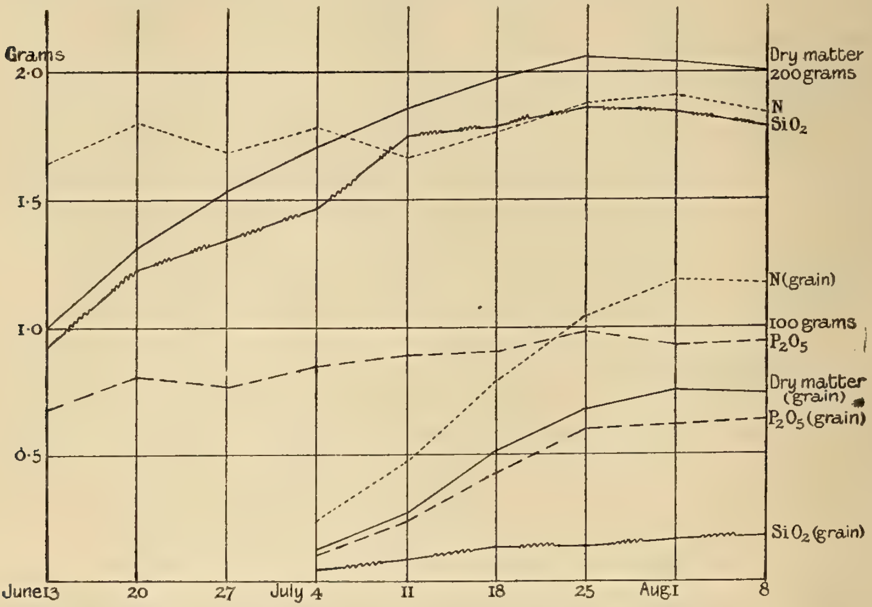

Fig. 2.—Dry Matter, Nitrogen, Phosphoric Acid, and Silica in Whole Plant and Grain. Means calculated on smoothed weights of whole Plant. ($SiO_2$ on ½ scale of N and $P_2O_5$.)

From these curves it will be seen that the dry matter of the crop goes on increasing until about a fortnight before cutting, but the whole of the nitrogen would appear to have entered by July 11, a fortnight before there was any sensible grain. The phosphoric acid seems to reach its maximum at a slightly later date, and the figures for the silica, though subject to greater errors of determination, show that the assimilation of silica continues still later, until the grain has progressed somewhat. Of the nitrogen within the plant, about 63 per cent. is eventually moved into the grain and rather less than 70 per cent. of the phosphoric acid: the migration of the phosphoric acid does not, however, take place exactly *pari passu* with that of the nitrogen, but follows it somewhat. Of the silica but a small proportion, 9 per cent. at the maximum, reaches the grain, and nearly the whole of this is transferred in the earlier stages of grain formation, being doubtless present in the adherent pales and glumes and not in the seed proper.

Table IV.—Hoos Field Barley. Season 1904.

Mean Weight and Composition of Barley (all Plots taken together).

| | | June 13. | June 20. | June 27. | July 4. | July 11. | July 18. | July 25. | Aug. 1. | Aug. 8. |
|---|---|---|---|---|---|---|---|---|---|---|
| Percentage (true means) in Dry Matter. | | | | | | | | | | |
| Grain | Nitrogen | — | — | — | 2·224 | 1·808 | 1·550 | 1·538 | 1·590 | 1·576 |
| | Phosphoric acid | — | — | — | 0·976 | 0·911 | 0·845 | 0·883 | 0·821 | 0·866 |
| | Silica | — | — | — | 0·919 | 0·722 | 0·540 | 0·412 | 0·428 | 0·443 |
| Straw and roots | Nitrogen | 1·642 | 1·372 | 1·101 | 0·963 | 0·745 | 0·664 | 0·605 | 0·552 | 0·530 |
| | Phosphoric acid | 0·675 | 0·609 | 0·492 | 0·464 | 0·407 | 0·314 | 0·281 | 0·235 | 0·234 |
| | Silica | 1·832 | 1·873 | 1·743 | 1·765 | 2·072 | 2·223 | 2·483 | 2·618 | 2·572 |
| Weights calculated on Smoothed Weights of Dry Matter. | | | | | | | | | | |
| Total dry matter (smoothed weights adopted) | | 100 | 131 | 153 | 170 | 185 | 197 | 205 | 203 | 200 |
| Dry grain | | — | — | — | 10·9 | 26·1 | 50·0 | 67·2 | 74·5 | 73·6 |
| Dry straw and roots | | 100 | 131 | 153 | 159·1 | 158·9 | 147·0 | 137·8 | 128·5 | 126·4 |
| Grain | Actual nitrogen | — | — | — | 0·242 | 0·472 | 0·775 | 1·034 | 1·185 | 1·160 |
| | ,, $P_2O_5$ | — | — | — | 0·106 | 0·238 | 0·423 | 0·594 | 0·612 | 0·637 |
| | ,, $SiO_2$ | — | — | — | 0·100 | 0·188 | 0·270 | 0·277 | 0·319 | 0·326 |
| Straw and roots | Actual nitrogen | 1·642 | 1·797 | 1·685 | 1·532 | 1·184 | 0·976 | 0·834 | 0·709 | 0·670 |
| | ,, $P_2O_5$ | 0·675 | 0·798 | 0·753 | 0·738 | 0·647 | 0·462 | 0·387 | 0·302 | 0·296 |
| | ,, $SiO_2$ | 1·832 | 2·453 | 2·667 | 2·808 | 3·292 | 3·268 | 3·422 | 3·363 | 3·250 |
| Whole plant | Actual nitrogen | 1·642 | 1·797 | 1·685 | 1·774 | 1·656 | 1·751 | 1·868 | 1·894 | 1·830 |
| | ,, $P_2O_5$ | 0·675 | 0·798 | 0·753 | 0·844 | 0·885 | 0·885 | 0·981 | 0·914 | 0·933 |
| | ,, $SiO_2$ | 1·832 | 2·453 | 2·667 | 2·908 | 3·480 | 3·538 | 3·699 | 3·682 | 3·576 |

Perhaps the most important point brought out is that the grain establishes a particular composition at an early stage in its development, after which, although it continues to grow and increase in weight, it does not sensibly alter its composition. From July 18 onwards the percentage of nitrogen and the percentage of phosphoric acid in the grain remain approximately constant, though the grain gains a further 50 per cent. of its weight during the same period. Whatever chemical changes take place during the latter stages of ripening, they consist in the rearrangement of the minerals within the grain rather than in any progressive change in the character of the intake.

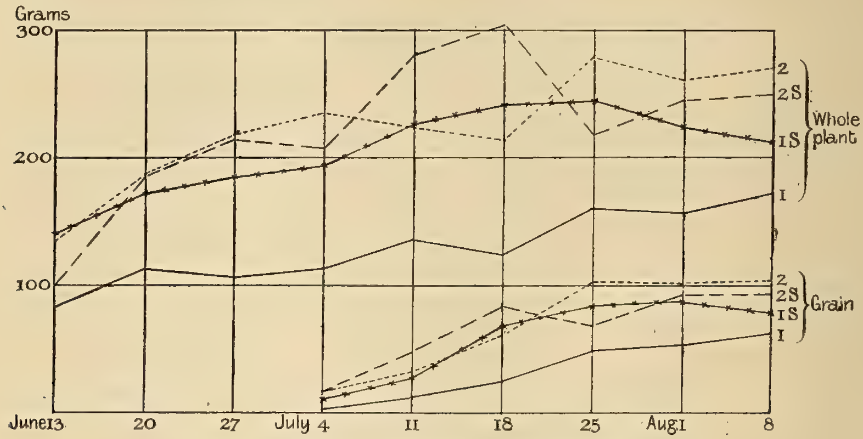

Fig. 3.—Dry Weights of plots without Potash.

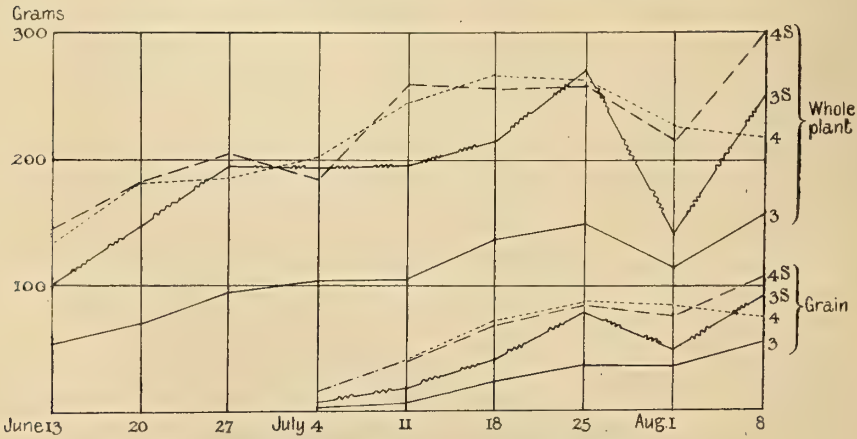

Fig. 4.—Dry weights of plots with Potash.

Taking these mean figures as indicating the normal course of development it will now be possible to review the results yielded by individual plots and trace the effect of silica on the assimilation of carbon (dry matter yield), nitrogen, and phosphoric acid, and particularly on the movement of these materials into the grain. Figs. 3 and 4 show in graphic form the yield from individual plots, fig. 3 deals only with Plots 1 and 2, where no potash is supplied in the manure, while fig. 4 deals with Plots 3 and 4, each of which receives equal amounts of sulphates of potassium, sodium and magnesium. In each figure curves are drawn separately for the silicated and non-silicated portions of the plot.

The accidental fluctuations in yield from week to week are too violent to admit of smoothing, but the general character of the curves shows that Plots 1 and 3, which receive neither phosphoric acid nor silica, give consistently a much lower yield than the others. The curves representing Plot 2 (with phosphoric acid), Plot 1 S (with silica), and Plot 2 S (with both phosphoric acid and silica), do not differ from one another by more than the extent of the accidental fluctuations from week to week of any one of them, but all indicate a yield about half as large again as that of Plot 1. Similarly where potash is used: Plot 3, without silica or phosphoric acid, never yields much more than half the crop on the Plots 3 S, 4, and 4 S, receiving either silica or phosphoric acid, or both together. As judged then by the dry matter produced, the silicate manuring is able to do the same work for the plant as the phosphatic manuring on Plots 3 and 4.

Despite the magnitude of the accidental fluctuations some differences in the character of the curves may be discerned; both Plots 1 and 3 (without silica or phosphoric acid) reach their maximum only on August 8, whereas in five of the other six cases where silica and phosphoric acid form part of the manure the maximum is reached by July 18 or 25. This would confirm the appearance in the field of deferred maturity in the absence of either phosphoric acid or silica.

Fig. 5 shows the proportion the grain bears to the whole plant at weekly intervals for the four plots which receive no potash, together with the smoothed mean of all the plots for comparison. It will at once be seen that on Plot 1, receiving neither phosphoric acid nor silica, the proportion of grain is below the normal, and also that the grain is later in forming. The 3 per cent. or so indicated on July 4, the earliest date when any separation of grain was possible, would be wholly made up of the adherent pales. It is only in the following week that the weight of grain has become sensible on Plot 1. On Plot 2, receiving phosphoric acid, the formation of grain precedes, and also is finally somewhat above the normal.

Plot 1 S, receiving silica but not phosphoric acid, occupies an intermediate position; though starting a little later than Plot 2, it eventually becomes almost identical with it. In other words, the free supply of silica without phosphoric acid to Plot 1 S has enabled the plant to mature as high a proportion of grain, and almost as rapidly, as does the supply of phosphoric acid to Plot 2. The further addition of silica to phosphoric acid as on Plot 2 S does not effect any change in the character of the development of the grain.

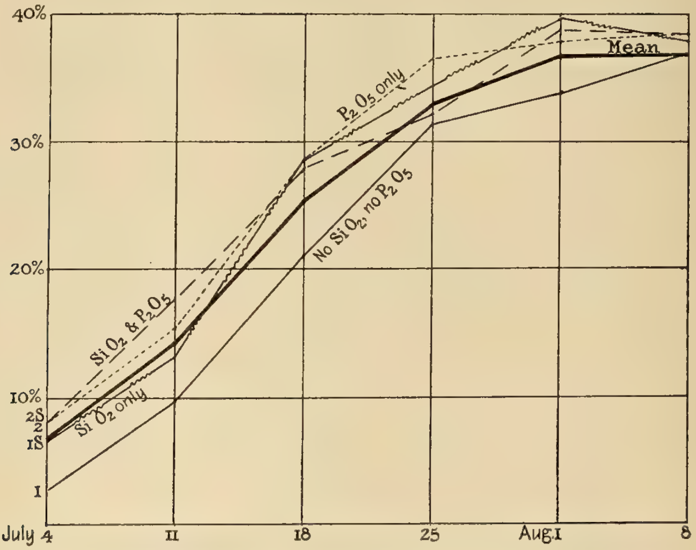

FIG. 5.—Percentage of Grain in Plant. Plots without Potash.

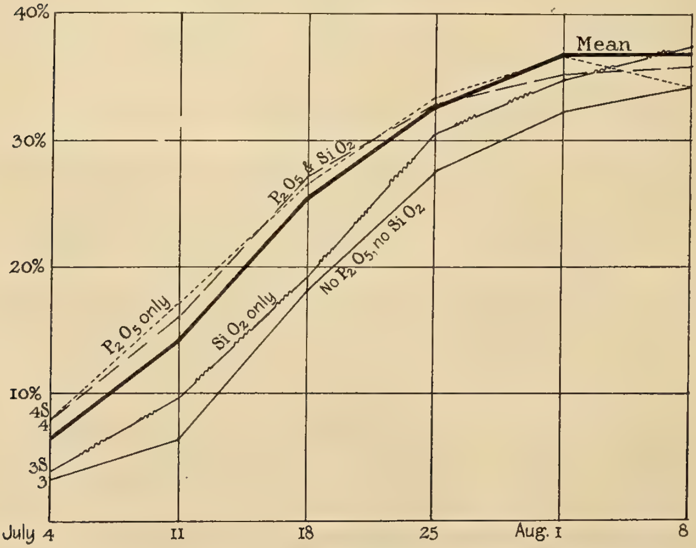

FIG. 6.—Dry Grain in 100 Total Dry. Plots with Potash.

In fig. 6 curves are seen representing the same succession of plots, this time however they all receive potassic manure. Again, the proportion of grain on the plot without either phosphoric acid or silica, 3, is low, and its formation is retarded as compared with the normal. Plots 4 and 4 S, the two plots receiving phosphoric acid, are practically identical and agree closely with the normal, while the curve representing Plot 3 S, where silica but no phosphoric acid is used, occupies an intermediate position. The development of grain on these plots receiving potash is later than is normal, though ultimately as high a proportion of grain to straw is produced.

As regards the formation of grain, the curves show that phosphoric acid hastens the formation of grain, and eventually causes a higher proportion of the material in the plant to pass over into that state, while silica acts in the same direction, though not to so large an extent.

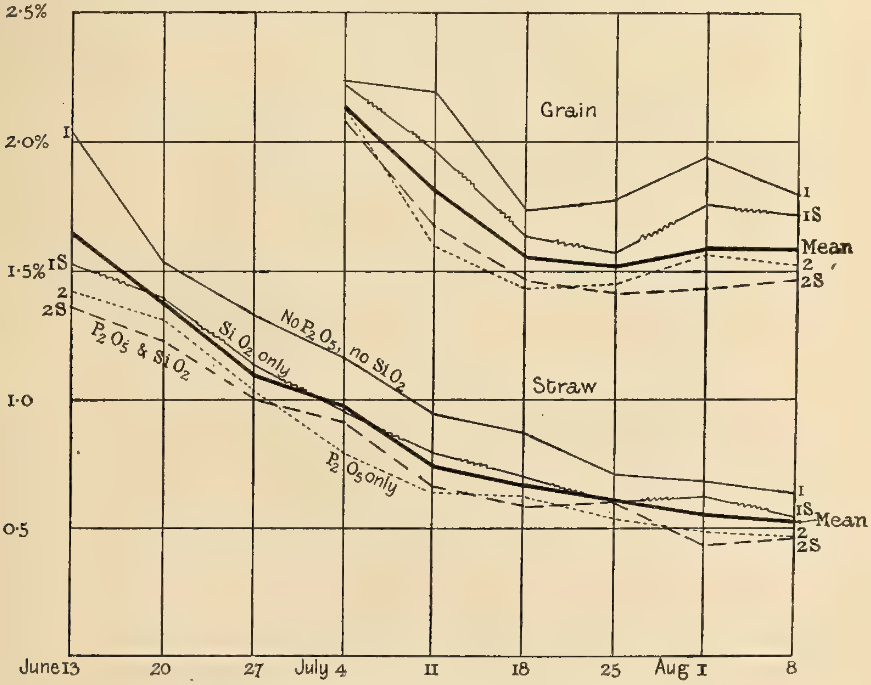

Fig. 7.—Percentages of Nitrogen in Dry Matter.

Turning to the entry of the nitrogen, fig. 7 shows the percentage of nitrogen in the grain and straw at the successive dates and for the four plots receiving no potash, the mean results being also plotted for purposes of comparison. Plot 1, receiving neither phosphoric acid nor silica, yields the highest proportion of nitrogen in both grain and straw at each stage of the growth. The use of phosphoric acid on Plot 2 reduces the percentage of nitrogen in both grain and straw to a little lower than normal level, and this reduction is most marked in the grain. Again, silica without phosphoric acid on Plot 1 S gives rise to an intermediate curve of development, nearer to the normal than to the curve representing the plot without either phosphoric acid or silica. Silica added to phosphoric acid (Plot 2 S compared with 2) makes practically no difference in the curve.

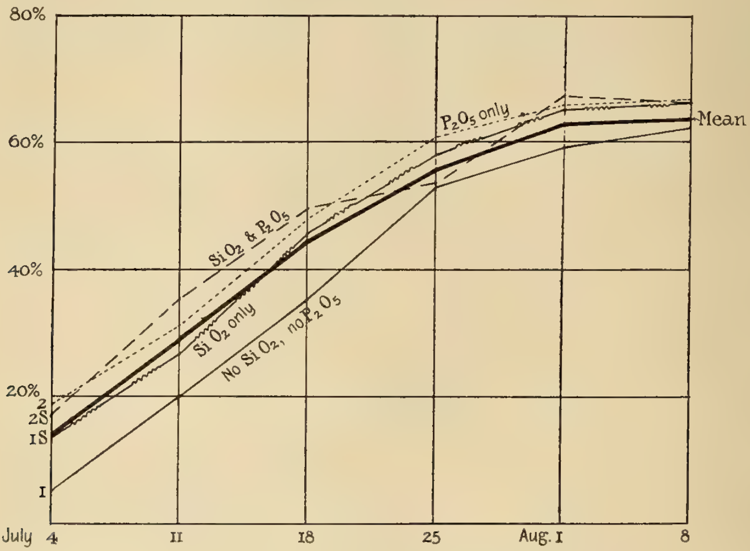

Fig. 8.—Nitrogen. Percentage of whole content present in the Grain. Plots without Potash.

Fig. 8 shows the movement of the nitrogen into the grain; although both the grain and straw of Plot 1, without phosphoric acid or silica, contain the highest percentages of nitrogen, yet the proportion of the nitrogen within the plant which passes over to the grain is lower on this plot than on the normal; the transfer again begins at a somewhat later date. The phosphoric acid alone on Plot 2 induces both an earlier and a greater proportionate transfer of nitrogen to the grain than the normal. Silica on Plot 1 S induces an earlier and more complete transfer of nitrogen, though not to the extent caused by the phosphoric acid. On the corresponding plots with potash (fig. 9) very similar results obtain; without phosphoric acid or silica (Plot 3) the transfer of nitrogen to the grain lags behind the normal, while the use of phosphoric acid (Plot 4) accelerates this process beyond the normal, silica (Plot 3 S) acts in the same direction though not to the same extent.

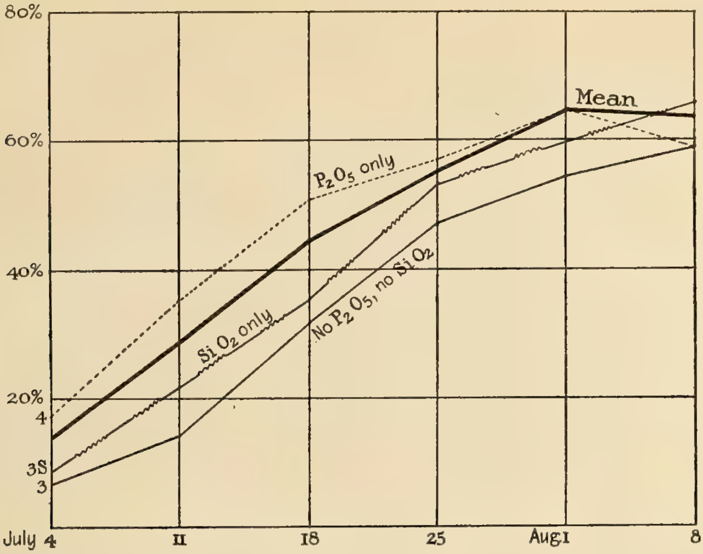

FIG. 9.—Nitrogen. Percentage of whole content present in Grain. Plots with Potash.]

As regards the phosphoric acid, the proportion of phosphoric acid in the dry matter of the grain is increased by the use of phosphatic manure, as it is also by the use of silica, especially where no phosphatic manuring takes place. The removal of the phosphoric acid to the grain is naturally more complete in the cases of phosphoric acid starvation; and when silica without phosphoric acid has been supplied, almost the whole of the extra

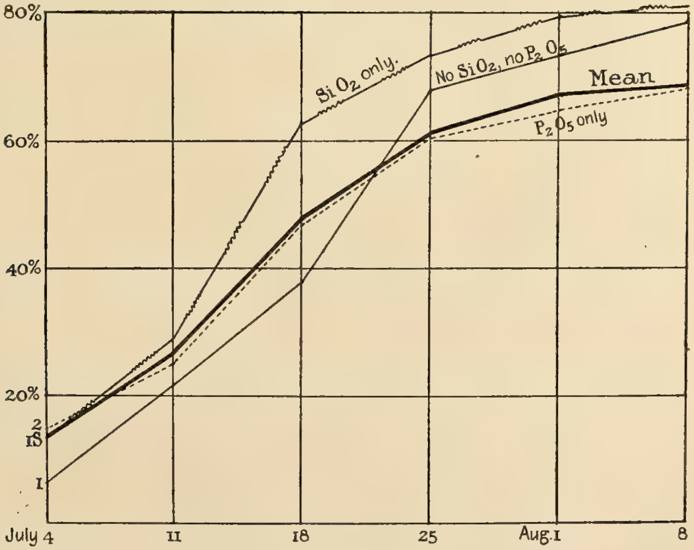

FIG. 10.—Phosphoric Acid. Percentage of whole content present in Grain. Plots without Potash.

phosphoric acid which the plant had thus been able to acquire is moved off into the grain. This may be seen more clearly in fig. 10, which shows what proportion of the plant's phosphoric acid is to be found in the grain on the successive dates. On Plot 1, without phosphoric acid or silica, the movement of phosphoric acid to the grain begins much later, but is ultimately more complete than on the normal or on the plots receiving phosphoric acid. With silica but no phosphoric acid (Plot 1 S) the migration of phosphoric acid begins at an earlier date and the proportion transferred is much increased, in spite of the fact that the actual amount of phosphoric acid in the plant is also much greater than on the first plot. Exactly the same conclusions are derived from an examination of the curves yielded by the parallel plots receiving potash (fig. 11); the use of silica both accelerates the migration of phosphoric acid to the grain and makes it more complete, although a greater proportion is initially present.

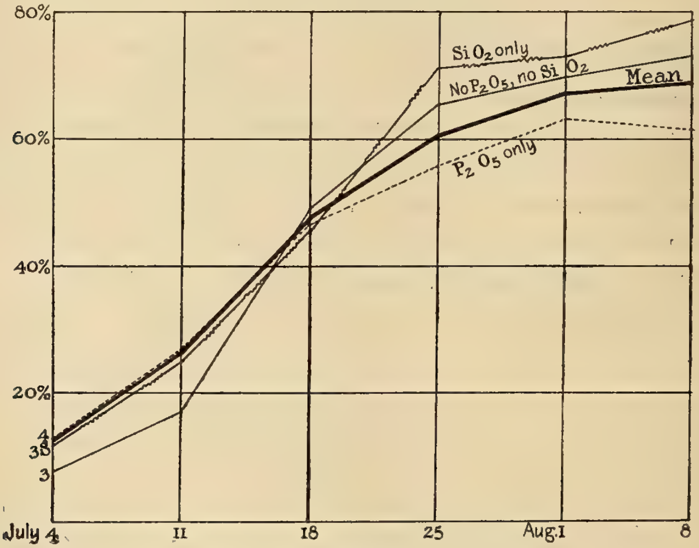

FIG. 11.—Phosphoric Acid. Percentage of whole content present in Grain. Plots with Potash.

The fact that a greater proportion of the phosphoric acid present in the plant is utilised in the grain on the silica plots, seems for the first time to indicate some specific action of the silica in facilitating the migration of phosphoric acid, so that it is not left unused as waste material in parts of the plant no longer active. But it will be found that the actual percentage of phosphoric acid finally left in the dry matter of the straw is no lower where silica has been used, on Plots 1 S and 3 S, than on the corresponding Plots 1 and 3 without either phosphoric acid or silica. If something like

0·11 per cent. of phosphoric acid be taken as the lower limit of phosphoric acid in the straw, that limit is just as much attained in the absence as in the presence of silica. The greater share of the plant's phosphoric acid transferred to the grain in the latter case comes from the fact that the amount of phosphoric acid assimilated, though increased by the silica, is still not sufficient for the requirements of the plant in the formation of grain, hence the straw continues to be depleted of its phosphoric acid to the lowest limit possible.

The consideration then of each of the factors submitted to detailed examination—the formation of grain and the migration of nitrogen and phosphoric acid into the grain—leads to the same general conclusion, that an abundant supply of soluble silica renders the barley plant more able to obtain a stock of phosphoric acid from the soil. On the plots therefore which are suffering from phosphoric acid starvation the manuring with sodium silicate acts like a supply of phosphoric acid; indeed, the plant does actually thereby obtain a larger amount of phosphoric acid.

Further evidence that the silica acts by stimulating the plant to take up phosphoric acid is derived from water cultures grown in 1904. Three plants of barley were grown in each of four jars, holding about 3 litres of solution, containing the following nutrient salts per litre:—

| | |
|---|---|
| Calcium nitrate ........................ | 1·64 gram. |
| Di-hydrogen potassium phosphate ... | 0·29 „ |
| Magnesium sulphate (crystallised)... | 0·62 „ |
| Potassium chlorate ..................... | 0·71 „ |

with a trace of ferric chloride.

Growth was vigorous from the first; the barley plants tillered freely and made a large number of shoots from each grain. On June 7 the nutrient solution was replaced by distilled water, which was changed again on the 8th, and replaced on the 9th by a fresh solution. The new solution contained calcium nitrate, magnesium sulphate, and potassium chloride as before in all the jars; the phosphoric acid, however, was varied as follows:—

No. 1. No phosphoric acid.
No. 2. No phosphoric acid, but 0·146 gramme silica in solution.
No. 3. 0·355 gramme phosphoric acid, no silica.
No. 4. 0·355 gramme phosphoric acid, and 0·146 gramme silica in solution.

It soon became evident that the phosphoric acid and silica, both separately and together, had a ripening effect, which was indicated by an earlier and an increased formation of ears.

On August 5, although the plants were by no means fully mature, it was necessary to harvest them because of an attack of aphis. When dried they gave the following results:—

Table V.—Barley in Water Cultures. Yield on August 5, 1904.

| Plot. | Number of ears. | Number of grains. | Dry matter. | | |
|---|---|---|---|---|---|
| | | | Grain. | Straw and roots. | Total. |
| | | | grammes. | grammes. | grammes. |
| 1 | 4 | 0 | 0 | 35·17 | 35·17 |
| 2 | 7 | 5 | 0 | 31·87 | 31·87 |
| 3 | 18 | 177 | 5·26 | 50·79 | 56·05 |
| 4 | 27 | 272 | 9·63 | 56·26 | 65·89 |

Assuming that on June 9, when the treatment was varied, all of the plants were approximately equal, it will be seen that the extra phosphoric acid added to Nos. 3 and 4 allowed them to double their weight during the remaining period of growth. The silica alone added to No. 2 did little to enable the plant to make better use of the restricted amount of phosphoric acid already in the plant, for although the formation of ears seems to have been a little forwarded, the few grains that were produced possessed no sensible weight. When, however, silica is provided in the presence of phosphoric acid, No. 4 compared with No. 3, it brings about a considerable increase of growth and an accelerated formation of grain—just such a change, in fact, as would be brought about by an increased assimilation of phosphoric acid. In fact, these cultures demonstrate that although silica cannot replace phosphoric acid, nor even economise and make more effective a restricted supply already within the plant, it will stimulate the plant to assimilate a greater amount of phosphoric acid should that be obtainable from the medium in which the plant is growing. Hence, when applied to a silica plant on a soil impoverished in phosphoric acid, it has the same effect in increasing and accelerating the formation of fruit as would result from a direct application of phosphoric acid.

It might be supposed that the action takes place within the soil itself, that the sodium silicate in some way attacks the insoluble phosphates of the soil so as to render them more available for the plant, much as an application of lime or gypsum will liberate an increased supply of potash from the soil. On chemical grounds it is difficult to see how such an action should occur, nor do the results with water cultures bear out such a view. To obtain further evidence on this point, samples of soil from the eight plots in question were extracted (1) with strong hydrochloric acid and (2) with a 1 per cent. solution of citric acid. While there is no method of determining the real amount of plant food in the soil which is at the service of the crop, the latter method* gives comparative estimates which are of value when dealing with soils of the same type.

Table VI shows a series of determinations of the total phosphoric acid and of the phosphoric acid soluble in 1 per cent. citric acid solution.

Table VI.

| | Total phosphoric acid. | | Phosphoric acid soluble in 1 per cent. citric acid. | |
|---|---|---|---|---|
| | No silica. | With silica. | No silica. | With silica. |
| 1 | 0·097 | 0·096 | 0·0086 | 0·0067 |
| 2 | 0·194 | 0·199 | 0·0495 | 0·0721 |
| 3 | 0·092 | 0·089 | 0·0075 | 0·0094 |
| 4 | 0·179 | 0·183 | 0·0674 | 0·0743 |

Comparing the soils with and without silica, the use of silica has not affected the amount of total phosphoric acid; the greater draft it occasions year by year from the soil of Plots 1 and 3, which are not supplied with phosphoric acid, is barely visible as yet in the analyses.

The silica has also little or no effect on the phosphoric acid soluble in citric acid on the four Plots 1, 1 S, 3, and 3 S; but the amount going into solution is distinctly higher on Plots 2 S and 4 S than on Plots 2 and 4, all plots receiving phosphoric acid in the manure. It is not, however, on these plots, but on Plots 1 and 3 that the silica shows any effect on the crop, hence these determinations support the conclusion that the sodium silicate has no action upon the soil phosphates.

Though the seat of the action is thus transferred from the soil to the plant, it is by no means settled whether the stimulus which the silica gives to the plant to enable it to take up more phosphoric acid from the soil reserves is a general stimulus or a specific one confined to the phosphoric acid. In other words, does the presence of a free supply of soluble silica so invigorate the plant that it is enabled to repair any weak link in the chain of nutrition and get as need be more nitrogen, phosphoric acid, or potash from the soil, or is the beneficial effect confined to the phosphoric acid alone? It is chiefly towards the settlement of this point that the further experiments both with silica and non-silica plants are now being directed.

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* Dyer, 'Chem. Soc. Trans.,' vol. 65, 1894, p. 115.

The further question of the intimate mechanism by which the silica acts within the plant, and the nature of the chemical changes into which it enters to bring about the observed effects, cannot yet be raised. In the first place little is known of how the phosphoric acid itself acts; it is evident that it induces seed-formation and hastens maturity, but in what way it takes part in the cell processes is still doubtful. Some of the data accumulated in the present investigation may profitably bear discussion in this connection—it is evident, for example, that there is little or no interdependence between the phosphoric acid and the assimilation or migration of nitrogen, as has often been suggested. Again, the results would seem to indicate that a distinction must be drawn between physiological maturity and ripeness. The grains of a phosphoric acid starved Plot like No. 1 go through a ripening process, but they never approach to the composition, or even attain the appearance, of the truly mature grain on more normal Plots like 2 and 4. The grain from Plots 1 and 3, though ripe, has still many of the characters of immature grain. If the progress of the grain be judged by such factors as the percentage of nitrogen or the ratio of phosphoric acid to nitrogen, the grain early in its formation settles down to a standard composition correlated with the original supply of nutriment, and after this point has been reached it does not change its gross composition, though it is continually increasing in size and weight. For example, the grain of Plot 1, with its high percentage of nitrogen and low ratio of phosphoric acid to nitrogen, which might be taken as indicative of its generally immature character, shows no tendency as its grows and ripens to approximate in composition to the thoroughly mature grain of Plot 2. The later stages of ripening are without doubt attended by changes in the nature of both the carbohydrate and the proteid contents of the grain, which however are not apparent in the elementary analysis of the grain.

## *Conclusions.*

The following general conclusions have been reached in the course of this investigation:—

(1) Silica, though not an essential constituent of plant food, does play a part in the nutrition of cereal plants, like barley, which contain normally a considerable proportion of silica in their ash.

(2) The effect of a free supply of soluble silica manifests itself in an increased and earlier formation of grain, and is thus similar to the effect of phosphoric acid.

(3) The silica acts by causing an increased assimilation of phosphoric

acid by the plant, to which phosphoric acid the observed effects are due. There is no evidence that the silica within the plant causes a more thorough utilisation of the phosphoric acid that has already been assimilated, or itself promotes the migration of food materials from the straw to the grain.

(4) The seat of the action is within the plant and not in the soil.

## APPENDIX.

### Table VII.—Hoos Field Barley, 1904.

Actual Dry Weights of Grain and Total Plant.

| Date of sample. | No silica. | | | | With silica. | | | | Mean of all plots. |
|---|---|---|---|---|---|---|---|---|---|
| | Plot 1. Nitrogen only. | Plot 2. No potash. | Plot 3. No phosphate. | Plot 4. Complete. | Plot 1 S. Nitrogen only. | Plot 2 S. No potash. | Plot 3 S. No phosphate. | Plot 4 S. Complete. | |
| | | | | Whole Plant. | | | | | |
| | grammes. | grammes. | grammes. | grammes. | grammes. | grammes. | grammes. | grammes. | grammes. |
| June 13... | 84·5 | 134·5 | 53·1 | 135·3 | 140·4 | 101·5 | 109·4 | 146·7 | 113·2 |
| „ 20... | 112·0 | 187·5 | 70·1 | 180·6 | 163·0 | 186·3 | 146·9 | 164·9 | 151·4 |
| „ 27... | 107·9 | 218·5 | 96·5 | 186·3 | 184·7 | 216·4 | 195·1 | 202·0 | 175·9 |
| July 4... | 116·5 | 236·5 | 103·0 | 201·1 | 194·0 | 203·8 | 193·7 | 184·7 | 179·2 |
| „ 11... | 138·9 | 225·0 | 104·0 | 244·5 | 226·6 | 280·7 | 194·9 | 258·0 | 209·1 |
| „ 18... | 124·2 | 215·1 | 134·7 | 266·9 | 241·4 | 304·0 | 212·2 | 255·6 | 219·3 |
| „ 25... | 161·4 | 279·2 | 139·3 | 263·1 | 245·1 | 219·5 | 260·2 | 260·0 | 228·5 |
| Aug. 1... | 158·9 | 260·9 | 111·1 | 226·5 | 223·0 | 244·8 | 139·4 | 214·3 | 197·4 |
| „ 8... | 173·3 | 270·2 | 154·4 | 217·1 | 211·4 | 249·9 | 248·7 | 301·1 | 228·3 |
| | | | | Grain. | | | | | |
| July 4... | 3·2 | 18·8 | 3·3 | 15·8 | 12·5 | 16·4 | 7·6 | 14·6 | 11·5 |
| „ 11... | 13·5 | 34·9 | 6·8 | 41·7 | 29·5 | 49·6 | 18·9 | 41·4 | 29·5 |
| „ 18... | 26·2 | 61·0 | 24·5 | 71·0 | 67·9 | 85·0 | 40·3 | 69·1 | 55·6 |
| „ 25... | 50·4 | 102·0 | 38·6 | 87·7 | 84·0 | 70·6 | 80·5 | 85·4 | 74·9 |
| Aug. 1... | 53·8 | 99·0 | 35·8 | 83·3 | 88·3 | 94·9 | 48·7 | 75·5 | 72·4 |
| „ 8... | 63·8 | 103·6 | 52·7 | 74·2 | 80·4 | 95·6 | 92·7 | 108·0 | 83·9 |

## Table VIII.—Hoos Field Barley. Season 1904.

Percentage of Nitrogen in the Dry Matter.

| Date of sample. | No silica. | | | | With silica. | | | |
|---|---|---|---|---|---|---|---|---|
| | Plot 1. Nitrogen only. | Plot 2. No potash. | Plot 3. No phosphate. | Plot 4. Complete. | Plot 1 S. Nitrogen only. | Plot 2 S. No potash. | Plot 3 S. No phosphate. | Plot 4 S. Complete. |
| | | | | In Grain. | | | | |
| July 4... | 2·245 | 2·133 | 2·358 | 2·228 | 2·225 | 2·193 | 2·547 | 2·165 |
| " 11... | 2·198 | 1·604 | 2·009 | 1·742 | 1·963 | 1·670 | 2·242 | 1·743 |
| " 18... | 1·735 | 1·431 | 1·715 | 1·516 | 1·637 | 1·460 | 1·739 | 1·480 |
| " 25... | 1·769 | 1·451 | 1·687 | 1·420 | 1·566 | 1·416 | 1·677 | 1·504 |
| Aug. 1... | 1·944 | 1·561 | 1·560 | 1·467 | 1·762 | 1·430 | 1·747 | 1·424 |
| " 8... | 1·791 | 1·517 | 1·578 | 1·461 | 1·716 | 1·463 | 1·733 | 1·448 |
| | | | | In Straw and Roots. | | | | |
| June 13... | 2·039 | 1·421 | 2·404 | 1·627 | 1·524 | 1·604 | 1·524 | 1·579 |
| " 20... | 1·541 | 1·309 | 1·551 | 1·268 | 1·398 | 1·234 | 1·465 | 1·413 |
| " 27... | 1·330 | 1·031 | 1·261 | 1·016 | 1·133 | 1·012 | 1·165 | 1·063 |
| July 4... | 1·162 | 0·797 | 1·151 | 0·977 | 0·957 | 0·909 | 1·121 | 0·812 |
| " 11... | 0·943 | 0·646 | 0·863 | 0·658 | 0·796 | 0·655 | 0·862 | 0·703 |
| " 18... | 0·868 | 0·617 | 0·824 | 0·535 | 0·704 | 0·580 | 0·747 | 0·624 |
| " 25... | 0·709 | 0·544 | 0·736 | 0·542 | 0·599 | 0·591 | 0·661 | 0·546 |
| Aug. 1... | 0·687 | 0·498 | 0·626 | 0·476 | 0·626 | 0·444 | 0·640 | 0·537 |
| " 8... | 0·633 | 0·472 | 0·573 | 0·528 | 0·538 | 0·467 | 0·542 | 0·533 |

## Table IX.—Hoos Field Barley. Season 1904.

Percentage of Phosphoric Acid in Dry Matter.

| Date of sample. | No silica. | | | | With silica. | | | |
|---|---|---|---|---|---|---|---|---|
| | Plot 1. Nitrogen only. | Plot 2. No potash. | Plot 3. No phosphate. | Plot 4. Complete. | Plot 1 S. Nitrogen only. | Plot 2 S. No potash. | Plot 3 S. No phosphate. | Plot 4 S. Complete. |
| | | | | In Grain. | | | | |
| July 4... | 0·591 | 1·064 | 0·746 | 0·869 | 0·861 | 1·092 | 1·116 | 1·007 |
| " 11... | — | 0·908 | 0·787 | 0·872 | 0·946 | 0·932 | 0·932 | 0·911 |
| " 18... | 0·403 | 0·889 | 0·736 | 0·857 | 0·814 | 0·937 | 0·889 | 0·893 |
| " 25... | 0·572 | 0·923 | 0·693 | 0·962 | 0·845 | 1·007 | 0·879 | 0·959 |
| Aug. 1... | 0·631 | 0·945 | 0·591 | 0·898 | 0·669 | 0·872 | 0·827 | 0·928 |
| " 8... | 0·650 | 0·972 | 0·638 | 0·990 | 0·776 | 0·961 | 0·820 | 0·942 |
| | | | | In Straw and Roots. | | | | |
| June 13... | 0·394 | 0·700 | 0·369 | 0·776 | 0·613 | 0·887 | 0·555 | 0·835 |
| " 20... | 0·362 | 0·667 | 0·386 | 0·713 | 0·576 | 0·692 | 0·490 | 0·739 |
| " 27... | 0·306 | 0·586 | 0·308 | 0·553 | 0·442 | 0·603 | 0·383 | 0·556 |
| July 4... | 0·256 | 0·543 | 0·294 | 0·511 | 0·401 | 0·654 | 0·342 | 0·545 |
| " 11... | 0·230 | 0·497 | 0·261 | 0·483 | 0·356 | 0·483 | 0·301 | 0·474 |
| " 18... | 0·180 | 0·404 | 0·173 | 0·364 | 0·193 | 0·416 | 0·249 | 0·392 |
| " 25... | 0·123 | 0·352 | 0·140 | 0·379 | 0·163 | 0·416 | 0·161 | 0·408 |
| Aug. 1... | 0·120 | 0·317 | 0·121 | 0·308 | 0·112 | 0·294 | 0·165 | 0·317 |
| " 8... | 0·105 | 0·283 | 0·122 | 0·322 | 0·116 | 0·301 | 0·134 | 0·364 |

Table X.—Hoos Field Barley. Season 1904.

Percentage of Silica in Dry Matter.

| Date of sample. | No silica. | | | | With silica. | | | |
|---|---|---|---|---|---|---|---|---|
| | Plot 1. Nitrogen only. | Plot 2. No potash. | Plot 3. No phosphate. | Plot 4. Complete. | Plot 1 S. Nitrogen only. | Plot 2 S. No potash. | Plot 3 S. No phosphate. | Plot 4 S. Complete. |
| In Grain. | | | | | | | | |
| July 4... | 0·331 | 0·920 | 0·344 | 0·730 | 0·876 | 1·216 | 0·657 | 1·236 |
| „ 11... | — | 0·680 | 1·016 | 0·620 | 0·800 | 0·712 | 0·696 | 0·777 |
| „ 18... | 0·335 | 0·442 | 0·501 | 0·621 | 0·466 | 0·631 | 0·486 | 0·630 |
| „ 25... | 0·294 | 0·329 | 0·448 | 0·336 | 0·484 | 0·536 | 0·384 | 0·493 |
| Aug. 1... | 0·301 | 0·403 | 0·382 | 0·388 | 0·433 | 0·521 | 0·337 | 0·558 |
| „ 8... | 0·339 | 0·475 | 0·339 | 0·417 | 0·443 | 0·548 | 0·419 | 0·468 |
| In Straw and Roots. | | | | | | | | |
| June 13... | 1·541 | 1·523 | 1·619 | 1·516 | 1·850 | 2·686 | 1·937 | 1·969 |
| „ 20... | 1·506 | 1·515 | 1·764 | 1·596 | 2·161 | 2·487 | 1·932 | 1·843 |
| „ 27... | 1·242 | 1·502 | 1·299 | 1·259 | 1·762 | 2·391 | 1·881 | 2·084 |
| July 4... | 1·191 | 1·528 | 1·368 | 0·907 | 1·988 | 2·784 | 1·767 | 2·257 |
| „ 11... | 1·415 | 1·974 | 1·604 | 1·578 | 2·516 | 2·527 | 2·124 | 2·280 |
| „ 18... | 1·643 | 1·966 | 1·404 | 1·569 | 2·656 | 2·905 | 2·388 | 2·558 |
| „ 25... | 1·809 | 2·214 | 2·085 | 1·954 | 2·678 | 3·069 | 2·462 | 3·291 |
| Aug. 1... | 2·210 | 2·203 | 1·858 | 1·915 | 2·985 | 3·550 | 2·644 | 3·171 |
| „ 8... | 1·572 | 1·690 | 2·356 | 2·033 | 3·028 | 3·874 | 2·748 | 2·923 |

# *On Innervation of Antagonistic Muscles. Ninth Note.—Successive Spinal Induction.*

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(Received January 31,—Read February 15, 1906.)

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It was previously* pointed out that in various reflex reactions inhibition is succeeded by marked exaltation of activity in the arcs inhibited. This after-effect may be figured as a sort of rebound from inhibition.

An example is the following. When a dog in which the spinal cord has been transected in the thoracic region is, the period of shock having passed, supported so that its spine is vertical and its hind limbs hang freely, these latter begin to perform a rhythmic stepping movement. This is the reflex, termed by Goltz the mark-time reflex. The tempo of this stepping differs, in my experience, in different dogs and at different times in the same dog. It may be as frequent at 22 steps of each leg per 10 seconds or as slow as seven steps in that period. It will persist in some animals for 20 minutes at a time. After some minutes' duration its amplitude usually becomes less and the movement on the whole less regular. For the first minutes of duration it is however regular and shows little variation.

The stimulus which excites this reflex has not been traced with exactitude. It persists after severance of the sciatic trunk not including the hamstring nerve. Freusberg† inclined to attribute it to afferents belonging to the "muscular sense," and especially to those connected with parts put under strain in the passive attitude given to the limb under its own weight. It is closely similar to the stepping reflex studied by Philippson‡ in the dog supported with spine horizontal. That it is initiated by the stretch of some tissue above the knee and especially on the flexor aspect of the hip may be argued from its immediate cessation when the dependent limb is supported from drooping by lifting the lower end of the thigh slightly from underneath by a prop placed just above the knee. Such support, in my experience, usually causes cessation of the reflex in the unsupported (fig. 1) as well as in the supported limb and it does not matter which of the two limbs is supported. The main stimulus, therefore, seems bilateral in origin, and to lie above the knee on the flexor aspect of the hip. The attachment of small weights to the foot has not, in my experience, increased the reflex.

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* 'Roy. Soc. Proc.,' B, vol. 76, p. 160.

† 'Pflüger's Archiv,' vol. 8.

‡ Heger's 'Travaux de Laboratoire,' Bruxelles.

In the stepping reflex obtained when the animal is supported vertically (the "mark-time" reflex) the movement is more pronounced at hip than at knee and ankle. A very similar stepping reflex occurs also when the animal is nearly supine. In this latter the movement is more marked at ankle and knee than at hip. In this posture of the animal passive dorsi-flexion of one ankle often excites dorsi-flexion of the opposite ankle, followed by extension at that knee and then by plantar-flexion at that ankle.

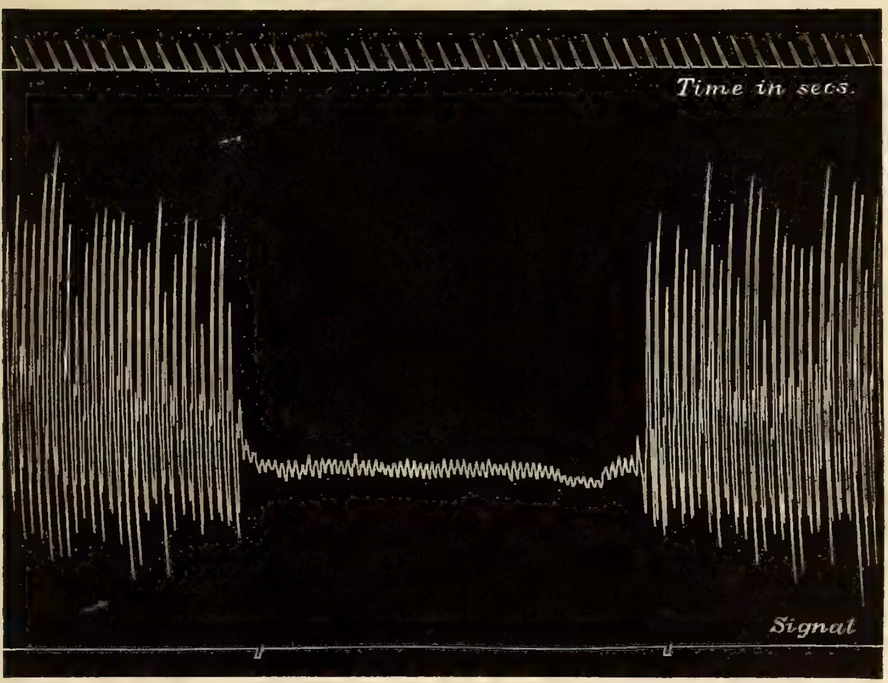

FIG. 1.—"Mark-time" reflex of spinal dog. The up strokes correspond with flexions of the limb, the down strokes with extensions. For the period between the two marks on the signal line the reflex was interrupted by taking the limb's weight off the fellow limb to that yielding the record, namely by supporting it under the knee. On return of the reflex, when the limb was again allowed to hang under its own weight, the reflex shows no increase beyond its previous activity. The small undulations during the period of rest are due to slight swaying of the animal; the reflex ceased completely. Time is registered above in seconds.

There is also a stepping reflex elicitable from the spinal dog when lying on its side and without any marked mechanical strain either of flexion or extension; this is obtained by faradisation of the skin of the opposite hind foot.*

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* Sherrington, 'Journ. of Physiol.,' vol. 33.

Here the tempo of the stepping is also about 20 steps per 10 seconds, but the stepping is strictly unilateral.

These points argue that several sources of excitation probably co-operate in the production of the stepping reflex. An important item in the execution of the movement of the reflex in all its forms is flexion at the hip and knee.

Suppose the "mark-time" reflex is in regular progress and is being recorded from one knee, *e.g.*, right by a thread passing thence to a pulley and light lever, if then the other thigh (left) be gently supported from behind the knee the record shows that the stepping reflex usually at once ceases in the right limb (fig. 1. The reflex ceases entirely: the small undulations on the trace in the interval during the cessation are due to swaying of the body, partly respiratory, in the suspended attitude). The limb during the cessation of the reflex hangs somewhat extended. On removing the slight support from under the left knee the "mark-time" reflex at once recommences, with flexion in the right knee. The reflex, on recommencing after this pause, continues as it ceased, that is, its tempo and amplitude are practically the same as before the interruption (fig. 1).

This result contrasts with the following. Goltz and Freusberg* showed that the "mark-time" reflex can be cut short by a strong squeeze of the tail. In my experience this stimulus is best applied near the root of the tail. A light touch on the hair of the tail often increases the stepping reflex, and the stronger the mechanical stimulus to the tail the quicker and more powerful as a rule is the inhibition of the stepping. But the stimulus to the tail need not be very strong in order to cause inhibition. I judge that the intensity of the mechanical stimulus which, applied to the tail, inhibits the reflex stepping is such that, were the condition of the animal not spinal, would constitute a dolorous (pathic) stimulus. The tail stimulus which inhibits may, therefore, be considered adequate for a nociceptive reaction.†

The application of this stimulus to the tail does not in any way interfere mechanically with the stepping movement. Suppose the "mark-time" reflex to be in regular progress and recorded as before, if then the tail stimulus be applied the stepping reflex is almost immediately arrested, and in both limbs. The reflex remains in abeyance while the tail stimulus is continued. On the cessation of the latter the reflex returns, and on its return soon shows indubitable increase in activity as compared with its activity before the inhibitory arrest (fig. 2). The increase is chiefly seen in the amplitude of the movement, but there is also often marked quickening of the tempo of the rhythm. I have seen the rhythm on some occasions quickened by

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* 'Pflüger's Archiv,' vol. 8.

† 'Journ. of Physiol.,' vol. 30, p. 39, 1903.

30 per cent. The after-increase of the reflex may persist in evidence for many seconds. Its decline is gradual.

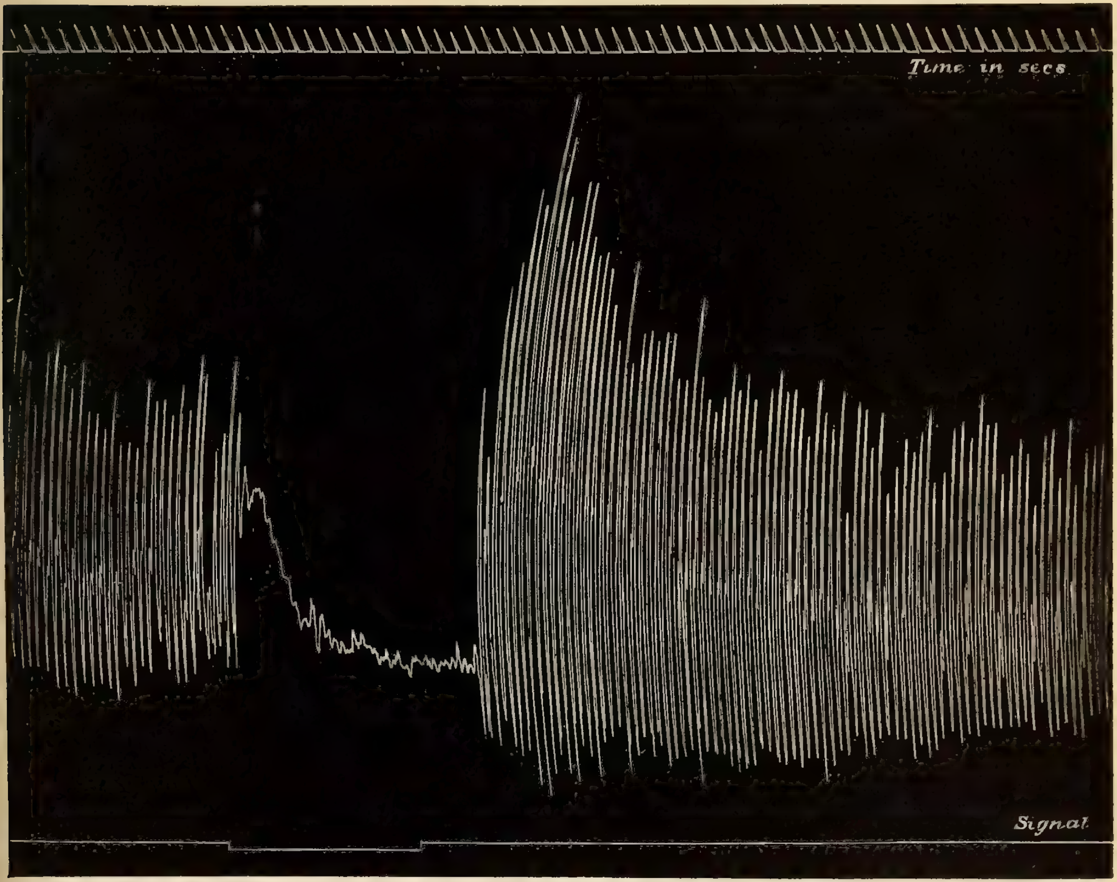

Fig. 2.—"Mark-time" reflex as before; but the reflex is here interrupted by stimulation of the tail. This arrest, due to *inhibition*, is followed, after cessation of the inhibitory stimulus, by increase in amplitude and slightly in frequence of the reflex. The signal registers period of application of inhibitory stimulus. Time registered in seconds.

The arrest of the stepping reflex by tail inhibition cannot be prolonged indefinitely. The reflex tends to return in spite of the inhibitory stimulation when the latter is long persisted in. It is different when the stepping reflex is arrested by lifting one knee; the reflex does not then tend to break through the arrest, however long the latter be continued. In this form of arrest of the reflex the arrest seems referable simply to cessation of the stimulus which excites the reflex. In the case of arrest by tail inhibition the arrest seems referable to a central inhibition, the peripheral stimulus, E, excitatory of the reflex remaining in action all the time, though unable to produce the reflex owing to the intervening inhibition.

The after-increase which ensues, in the second form of arrest, but not in the first, might be explicable in either of two ways. It might be due to the continuance of the exciting stimulus, E, during the period of arrest. That stimulus might, though unable to evoke discharge of the motor neurone during the inhibition, yet be charging a relay apparatus in the reflex arc, and so lead to increased discharge after the inhibition was past. Or the after-increase might proceed as a direct result from the inhibition itself, the depressed activity of inhibition being followed by a rebound to super-activity, and altogether apart from the continuance of any excitatory stimulus during the inhibitory period.

To decide between these possibilities the effect of strongly stimulating the tail when at the same time both hind limbs were supported from below was tried. The stimulus for the stepping reflex was thus held in abeyance at the time of and during the whole period of the intercurrent inhibition. The result was found to be an after-increase of the stepping reflex not less marked than in the previous cases.

It is not at first obvious what relation a stimulus to the tail bears to the reflex of the limb. But it is often noticeable that in the "mark-time" reflex the tail itself is alternately deflected to right and left, keeping time with the stepping reflex. When the right limb begins to draw up in flexion, and the left limb to straighten out in extension, the tail begins to move from the right to the left. The tail does really therefore participate in the locomotor reflex, of which the stepping movement is also a part. Nocuous stimuli to the side of the tail, *e.g.*, by unipolar faradisation, evoke reflex abduction of the tail from the side stimulated, and the organ is then usually kept abducted for a time, just as the hind paw is drawn up and kept so for a time when excited by similar stimulation. Moreover, such stimulation of the tail excites reflex movement not only of the tail but of the hind limb, and the limb's movement is usually extension at hip and knee. The result of this is, that the tail stimulus can inhibit a flexion-reflex of the hind limb. If the flexion-reflex be induced by inserting a hedgehog spine into the *planta*, and if while that prolonged reflex is in progress and the limb is remaining thoroughly flexed at hip and knee, a caudal skin-point is faradised, the limb at once drops into the extended attitude under gravity. This occurs when the nerves to the extensors to the hip and knee have been severed. The tail stimulus therefore inhibits the flexors of knee and hip.

The particular mode in which the tail-stimulus comes to inhibit the stepping reflex seems to be that it inhibits the rhythmic flexion of the hip, which is so prominent a part of the mark-time reflex. The after-increase of the latter reflex following on its inhibition by the tail-stimulus seems, as shown above, a pure effect of rebound from inhibition. On the above view it should show itself therefore chiefly in an after-increase of the flexion of hip movement, and the graphic records of the effect show this to be the case (fig. 2), the movement of flexion being the upward movement in the tracing.

This inhibition of the "mark-time" reflex exemplifies therefore the *principle of the common path.*[*] The reflex arc whose reaction is inhibited and the reflex arc which inhibits are both found when separately examined to use the same final common path, but to different effect. The *common path* in this case is the flexor neurone of the hip, and one arc uses it in a steady depressor manner and the other in a rhythmic pressor manner. The conflict in this case, as so often, is between a nociceptive reaction and a purely locomotor reaction; and the former prevails as is usual.[†]

The after-increase consequent upon inhibition is evidently a form of "*bahnung.*"[‡] In order to distinguish it from those forms of "*bahnung*" which ensue without previous inhibition and are therefore immediate, it may be conveniently termed "*successive spinal induction,*" the more so as that term draws attention to the likeness between the spinal process and certain visual phenomena commonly designated "induction."

Another instance of "successive spinal induction" is the following: In the spinal animal (cat, dog) lying supine, the knee-jerk is elicited at regular interval by tapping the patellar tendon. If, then, the central end of the previously severed hamstring nerve is faradised, the knee-jerks become much less ample or quite inelicitable. The tonus of the knee-jerk muscle (vasto-crureus) is at the same time depressed. On discontinuing the stimulation of the hamstring nerve, the knee-jerk quickly becomes again elicitable, and soon is more brisk and ample than prior to the intercurrent inhibition[§] (fig. 3). The tonus also returns and in some cases becomes clearly greater than prior to the inhibition. This after-increase of the knee-jerk takes place when, during the whole period of inhibition, the leg is by mechanical support prevented from drooping, and thus the passive stretch of the vasto-crureus is avoided during the inhibition. The after-increase also occurs when the elicitation of the knee-jerk is completely remitted during the whole period of the inhibition. The after-increase is not therefore due to any continuance of the action of exciting stimuli during the period of inhibition. It is a "successive spinal induction" following upon inhibition, just as in the instance previously given.

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[*] Sherrington, 'Brit. Assoc. Reports,' 1904, Address to Section I.

[†] *Ibid.*

[‡] Exner, 'Pflüger's Archiv,' vol. 28.

[§] 'Roy. Soc. Proc.,' B, vol. 76, p. 161.

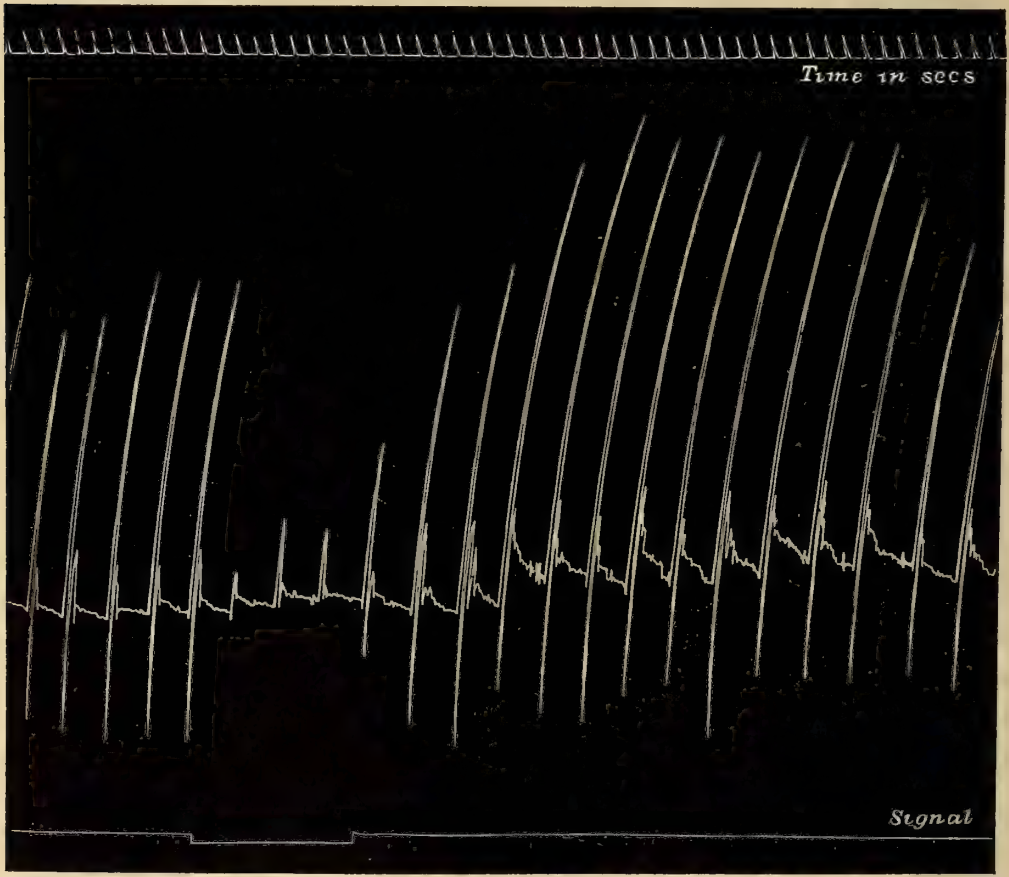

FIG. 3.—Knee-jerks. The knee-jerks were elicited by taps of equal intensity delivered at intervals signalled by a metronome. During the time marked by the signal the afferent nerve of a flexor muscle of the knee was weakly faradised. This inhibitory stimulus depressed the knee-jerk. After the inhibitory stimulus was discontinued the jerks increased to beyond their amplitude prior to the inhibition; this increase is accompanied by a tonic after-action following each jerk. Time registered above in seconds.

In the "scratch-reflex," after its inhibition by the crossed extension-reflex, or the homonymous flexion-reflex, a similar after-exaltation is sometimes seen. Fig. 4 exemplifies such an occurrence. But the time of interruption of the reflex has usually in my records been too short to allow much scope for the development of successive spinal induction, and the quick tiring of the scratch-reflex under electric excitation is unfavourable to examining it there.

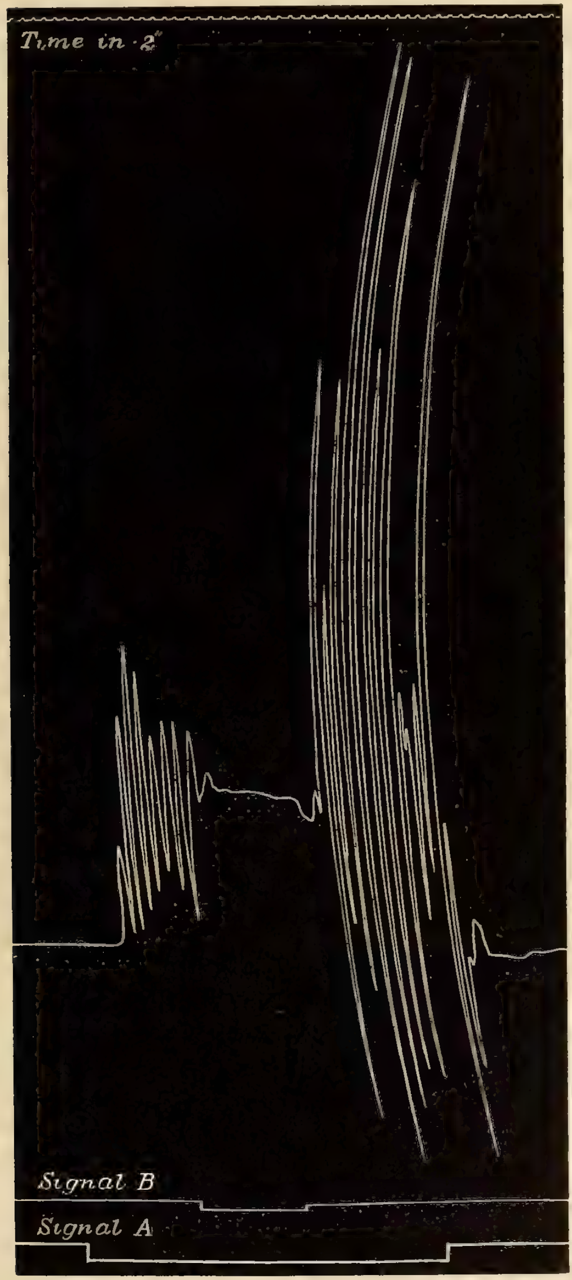

Fig. 4.—Scratch-reflex of spinal dog. The reflex was evoked from the skin of the shoulder by unipolar faradisation applied for a period marked by the signal line A. The reflex soon after its commencement was inhibited by stimulating (unipolar faradisation) the skin of the *planta* of the foot engaged in the scratching-reflex. The time of the inhibitory stimulus is registered by signal line B. After cessation of the inhibitory stimulus the scratch-reflex—its stimulus being continued throughout—returned, and on return was more ample than before the inhibition. Time registered above in fifths of second.

It is easy to evoke reflex-extension of the hind limb by stimulation of the skin of the opposite hind limb. With the spinal dog laid on its side (*e.g.*, left) and a thread attaching the knee of the slightly flexed right limb to a recording lever, the delivery of a certain stimulus (*e.g.*, 15 break-shock at 30 per second) by unipolar faradisation through a gilt needle at a skin-point of the left foot, evokes reflex-extension at right hip and knee. If this stimulus, at moderate and unchanged intensity, be given at regular interval (*e.g.*, once per minute) a series of extension reflexes of regular height and duration are obtained. If in the course of such a series the right limb is, during one of the 60-second intervals, thrown into strong reflex flexion (*e.g.*, by faradisation of the skin of its own foot and the reflex flexion be maintained for some time, *e.g.*, 40 seconds) the next extension-reflex following on the intercurrent flexion differs from those prior to it in being more ample and more prolonged (figs. 5 and 6). Its after-discharge is greatly increased and its latency is sometimes diminished. If the test stimulus for the extension-reflex be adjusted at just subliminal value, the intercurrent flexion-reflex will make it supraliminal. The exaltation of the extension-reflex may remain perceptible for five minutes; in the example furnished in fig. 5 it is quite recognisable for four minutes.

The mode of production of this exaltation seems the same as that traced above in the knee-extensor, with the knee-jerk as test-sign, and in the stepping reflex after interruption by tail inhibition. It has been shown that central inhibition of the extensor of the knee is part and parcel of the homonymous flexion-reflex of the leg. In the present case, therefore, during the intercurrent flexion-reflex, the reflex arc of extension was under inhibitory depression. After discontinuance of that inhibition the extensor reflex is found exalted to a degree of activity beyond that which it showed prior to the inhibition.

A similar successive spinal induction is evident in the following. The spinal animal (cat) being supine, the nerves of one hind limb are severed save for the nerve to *vasto-crureus.* The limb is supported with femur vertical and anticrus therefore fairly flexed at knee. The central end of the hamstring nerve is faradised; this causes the usual reflex inhibition of vasto-crureus, an inhibition which, if there be little or no tonus in that muscle at the time, is difficult to detect by mere inspection, though easily revealed by abolition of the knee-jerk. If the faradisation of the central end of the hamstring nerve be continued for some seconds, *e.g.*, three, on its cessation there often ensues a marked reflex extension of the knee. This is no mere return to previously existent slight tonus; it is a fairly intense contraction of the vasto-crureus, often sufficient to extend the knee fully and passing off again in three or four seconds' time. It sets in, in my experience, not at the very moment of withdrawal of the inhibitory stimulus, but in the course usually of the first three seconds following that withdrawal. A tap given to the patellar tendon appears sometimes to elicit it when otherwise it would not ensue spontaneously.

Related to this phenomenon seems the following. When a flexion reflex of the hind limb is by appropriate stimulation continued for a long time in the spinal dog, the flexion tends to be broken through* from time to time by short-lasting explosive extensions of the limb, much resembling the "extensor-thrust." In all these cases the extensor arc during the flexion-reflex has been under prolonged inhibition, and the superactivity which it shows under a test stimulus, the "spontaneous" discharge which it exhibits on relief from the inhibition, and the explosive outbreak which it gives when the inhibitory reflex is getting fatigued, all seem to be evidences of "successive spinal induction" supervening as a rebound after inhibition.

The effect upon the direct flexion-reflex of an intercurrent extension reflex is, in my experience, much less marked than the converse just described. This may be due in part to my having used the crossed extension-reflex and not a direct extension-reflex as the intercurrent reflex. The crossed reflex is less potent and powerful than the uncrossed reflex.† But the only homonymous extension-reflex of the limb available is the "extensor-thrust," and that is so unmanageable, and especially is so little capable of prolongation, that it was unsuited to this purpose. However, an influence can be traced, and in other ways than by intercurrent extension in a series of reflex flexions. Thus, with the spinal animal vertical, the hind limbs are taken and kept fully extended at hip, knee, and ankle; then usually, in a short time, a strong flexion-reflex at hip and knee supervenes. Again, if similarly one hind limb be strongly passively flexed at hip but strongly extended at knee and kept in that posture for a short time, it is usual for any attempt to passively extend the hip to elicit at once strong reflex contraction of the flexors of the hip, preventing passive extension.

Nevertheless, the greater inductive effect of flexion upon extension than of extension upon flexion as examined at the knee-joint, seems, in my experience, marked. In regard to it one remembers that though electrical stimulation of the afferent nerve-fibres from the flexor muscles has been shown to inhibit the reflex contraction of the extensor,‡ it has not been

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* 'Journ. of Physiol.,' vol. 34, p. 34, fig. 21, phase 3.

† Sherrington, 'Brit. Assoc. Reports,' *ibid.*

‡ 'Roy. Soc. Proc.,' vol. 52, p. 556.

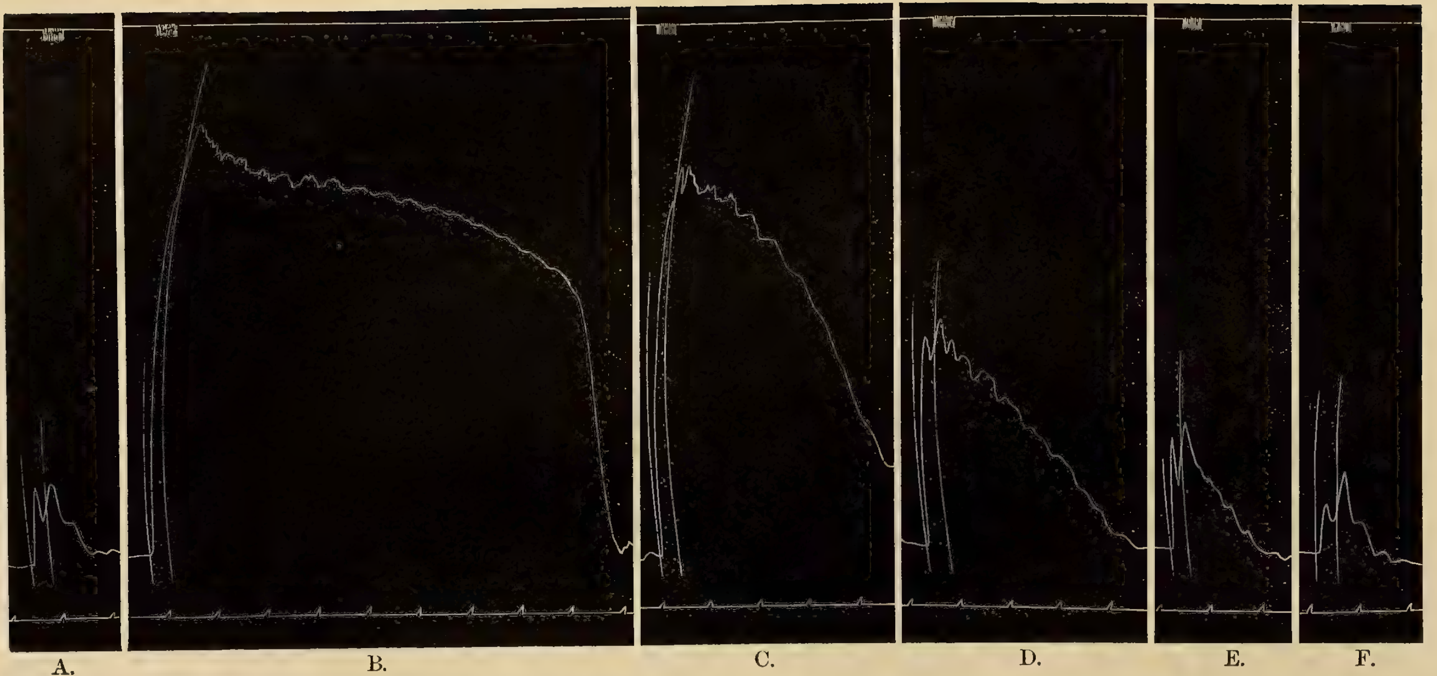

Fig. 5.—Crossed extension-reflex in the spinal dog. The reflex was evoked at one-minute intervals by equal series of break induced-currents applied unipolarly to the skin of a digit of the fellow limb to that yielding the record. The reflex was of low intensity, the induced currents being weak, and only 10 being delivered, so that the stimulus, at the rate chosen for the succession of the currents, lasted less than a half-second. Between stimulations A and B a strong flexion-reflex of the limb, namely of that limb entering into extension in the crossed extension-reflex, was provoked and maintained for 35 seconds. The extension reflex B following next after the intercalated flexion-reflex shows increase in amplitude and duration, especially in after-discharge. This increase subsides gradually, but is obvious in the three following reflexes C, D, and E, also elicited at one-minute intervals. In reflex F, evoked in the fifth minute after the intercalated flexion-reflex, the augmentation is not present, and the reflex intensity is about equal to that obtaining in the series, of which A is an example, elicited before the intercalation of the flexion-reflex. The signal above registers the series of break-shocks forming each stimulus. Time is signalled below in seconds. The intensity of the stimulus and the point of its application remained unaltered throughout the series of records.

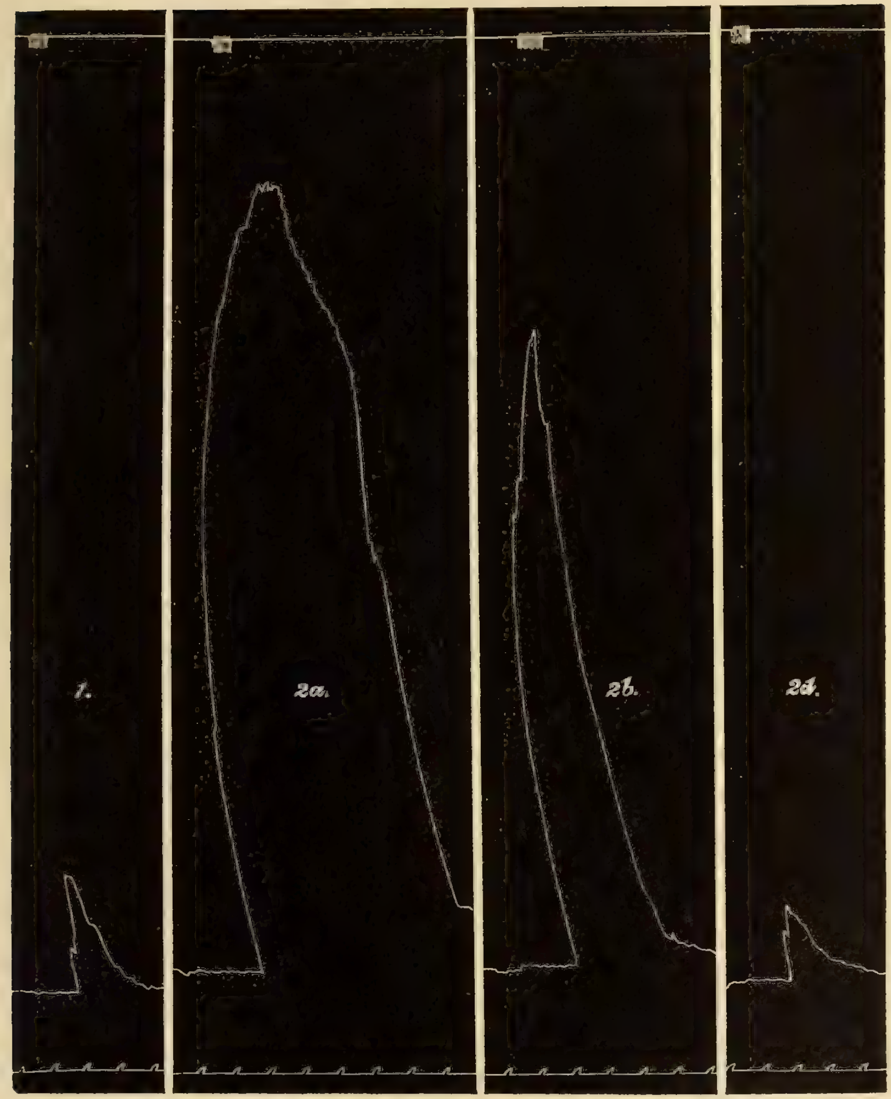

FIG. 6.—Crossed extension-reflex. The reflex was being elicited regularly by short series of break-shocks, the series being equal in intensity and duration. (In 2*b* nine shocks were delivered instead of eight in the other stimulations, owing to a defect in the rotating key.) Reflex No. 1 is the last of a series of equal reflexes thus provoked; both stimulus and reflex were of low intensity. After reflex No. 1 in the one-minute interval between it and 2*a*, a strong flexion-reflex of the limb was excited and maintained for 40 seconds. The next following reflex 2*a* exhibits augmentation, and this augmentation is obvious also in reflex 2*b*. Reflex 2*d* was elicited two minutes after 2*b*, and shows no augmentation. It is somewhat less than the reflex evoked just prior to the intercalated flexion-reflex. The signal above registers the time, etc., of the break-shocks used as stimuli. Time is marked below in seconds. The intensity of the stimulus and the place of its application remained unaltered throughout the series of observations.

shown conversely that similar stimulation of the afferent fibres of the extensor muscle (vasto-crureus) inhibits contraction of the flexor muscle. To examine this latter point is not altogether easy, since the nerve severance of the vasto-crureus nerve, in order to stimulate its central end, of necessity renders impossible the maintenance, let alone the examination, of any reflex status of that muscle. I have, however, succeeded in *splitting* the nerve, and if that is done without too much damage to either half of it, one division can be severed and dissected back into the psoas, and the other remain preserving the knee-jerk and, more important, a fair extensor tonus at the knee. The central end of the severed division can then be stimulated, and its effect on the rest of the extensor muscle itself be thus observed.

To employ electrical stimulation with as little risk of escape of current as possible it is necessary to cut the divided nerve as low as possible, and dissect it up into psoas as high as possible; in this way I have obtained sometimes 5 cm. length of free nerve. I have further exsected the internal saphenous nerve right up into the psoas, and also the external division of the muscular division of the anterior crural nerve, hoping thus to minimise current escape from the stimulated branch of vasto-crureus nerve to those other branches of the anterior crural. In a previous communication I discarded effects of electrical stimulation of the nerve of the extensor muscle as too open to error by escape of current.* Further examination with the above precautions makes me, however, regard the following as reliable reflex effects obtainable by stimulation of the central end of the vasto-crureus nerve itself: inhibition of the tonus of vasto-crureus itself, the tonus returning to some extent immediately on cessation of the stimulus if the stimulus be weak and brief; inhibition of the knee-jerk, contraction of rectus femoris, especially of its upper part, and tensor vaginæ femoris and psoas, dorso-flexion of ankle, some slight contraction of the hamstring muscles, especially deep inner hamstring, and extension of opposite knee with inhibition of its hamstring muscles. These effects obtained by faradic excitation are also obtainable, but in slighter measure, by drawing a ligature tight upon the central end of the nerve (mechanical stimulation). The total effect is flexion of the homonymous and extension of the opposite limb.

There is thus no evidence that the afferent nerve-fibres from this extensor muscle when excited in these ways inhibit contraction of the flexors, although the afferent fibres from the knee-flexor when similarly excited do inhibit contraction of this extensor. Reciprocal innervation is evident in the reflex effect obtained from the afferents of each muscle, for those of each inhibit one set of muscles and excite the muscles antagonistic to the inhibited group. But in each case the reciprocal innervation has the same direction, namely, excitation of the flexors and inhibition of the extensors. This relation would obviously tend to make it more facile for flexion of the limb to successively induce extension than for extension to induce flexion.

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* 'Roy. Soc. Proc.,' B, vol. 76, p. 283.

And another consideration has to be borne in mind. The measure to which the intercurrent flexion-reflex exalts the following crossed extension-reflex can be estimated in terms of the relation existing in the crossed extension-reflex between intensity of exciting stimulus and intensity of reflex response. A somewhat widely-expressed opinion is found in the literature dealing with reflex action to the effect that intensity of reflex response is relatively little determined by increase of intensity of exciting stimulus (Wundt,* Hallstén,† Biedermann,‡ Baglioni§). Observations by Merzbacher|| in the intact limb of the frog, and by Pari¶ in the isolated gastrocnemius, by myself** in the scratch-reflex, and by Langendorff†† in the flexion-reflex of the tortoise, show that in some spinal reflexes at least there can be obtained marked grading of intensity of reflex response in conformity with grading of intensity of stimulus. In the extension-reflex of the hind limb as obtained by stimulation of the opposite hind foot the amplitude of the movement and its duration increase with increase of intensity of the exciting stimulus. The relation between the intensity of the stimulus and that of the response in this reflex is, in my experience, somewhat different from that which obtains in several other reflexes, *e.g.*, in the direct flexion-reflex and in the scratch-reflex. The successive increments of intensity of stimulus cause increase of the extension-reflex by fairly gradual and regular degrees up to a certain point. Beyond that point relatively larger increments of reflex response result from increase in intensity of stimulation (fig. 7). This character of the ratio in this reflex between increment of stimulus and increase of response is especially evident with the after-discharge of the latter. In regard to the successive spinal induction exemplified by the reflex, it is clear that if the intensity of stimulus chosen for testing the crossed extension-reflex be near below that value at which

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* 'Untersuch. z. Mechanik d. Nerven u. Nervencentren.'

† 'Archiv f. Physiol.,' 1885—1888.

‡ 'Pflüger's Archiv,' vol. 80.

§ 'Verworn's Zeitschrift.'

|| 'Pflüger's Archiv.'

¶ 'Archives Italiennes de Biol.'

** 'Physiol. Soc. Proc.,' March, 1904.

†† 'Sitzungsb. d. Naturforsch. Versam.,' 1905.

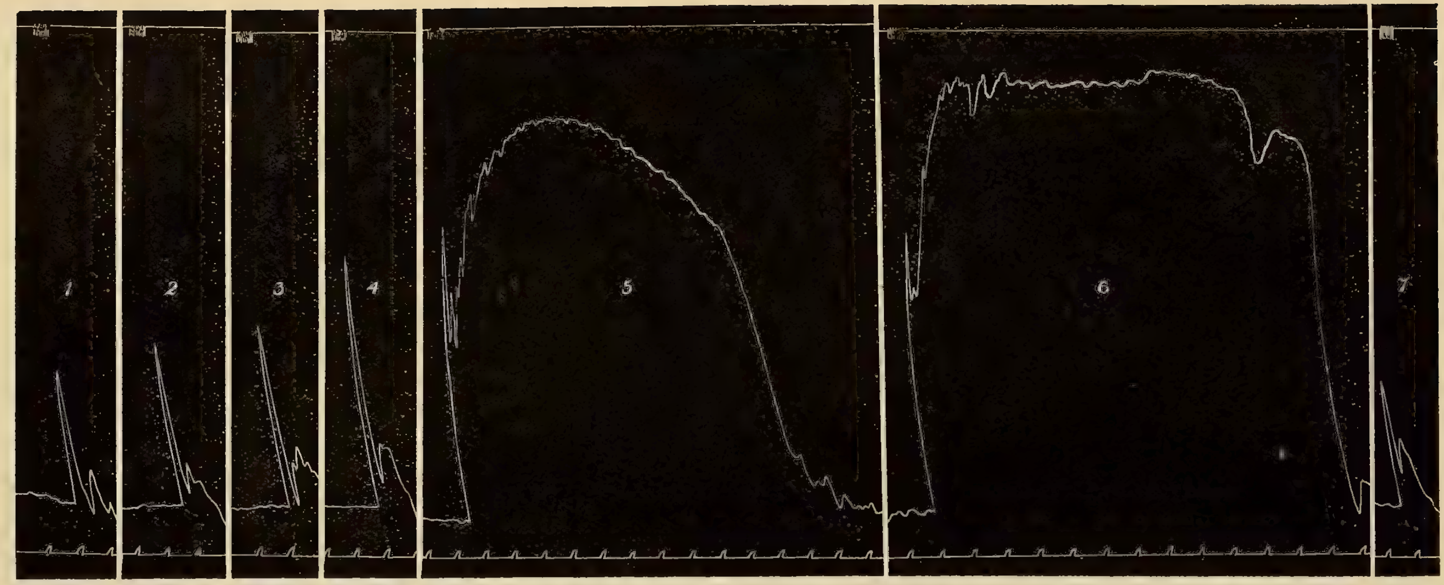

Fig. 7.—Crossed extension-reflex. Grading of intensity reflex corresponding with grading of intensity of stimulus. The time and duration of the stimulus are shown in the top line by the electro-magnet signal, in the primary circuit of the stimulating apparatus. For the elicitation of each reflex 12 break-shocks, at the rate of 25 per second, were delivered by a needle kathode to the skin of a digit of the opposite foot; the anode was diffuse and applied headward of the spinal transection. An interval of two minutes elapsed between each stimulation and the one next following it.

| | Intensity of stimulus. | Measure of reflex. |
|---|---|---|
| Reflex 1. | 475 | 3·5 |
| 2. | 560 | 5 |
| 3. | 690 | 7 |
| 4. | 890 | 9 |
| 5. | 1100 | 355 |
| 6. | 1400 | 459 |
| 7. | 560 | 4·5 |

The intensity of stimulus is given in units of the Kronecker inductorium scale; measure of reflex in units of area of the records registered in the tracing. Time below in seconds. The record is from the same animal as that yielding fig. 8.

further increment produces great increase of response, the exaltation induced by an intercurrent flexion-reflex need not be very extreme to give, nevertheless, a very apparent and great increase in the response. A not very extreme superactivity induced in the internal condition of the arc might suffice to give the external stimulus a value equivalent to a stimulus that would produce a very much greater reflex response.

In the homonymous flexion-reflex the increments of reflex response ensuing from increments of intensity of stimulus follow, in my experience, a more regular progressive increase (fig. 8) than in the crossed extension-reflex. There is therefore with this flexion-reflex less chance of successive spinal induction effecting an augmentation apparently so large as with the extension-reflex. This also has to be remembered, therefore, in contrasting the smaller effect observed in the induction of flexion by extension than of extension by flexion in the hind limb.

The linking together of the simpler reflexes which compose a usual reflex cycle doubtless involves several processes; it has attracted the attention of observers from several points of view. Loeb* has illustrated how in regard to segmental reflexes the effect of the reflex in one segment may be to transfer the external stimulus to another segment where it in turn excites the reflex of that segment, and so on further. In this way the reflex sequences, which he terms "Ketten-reflexe," can be compounded.

Another interesting connecting process welding simpler reflexes into more composite is that discovered by v. Uexküll.† He has shown that a piece of musculature, under static conditions which make it of greater length, is more prone to excitation through the nervous arcs than it is under conditions in which its length is less. Thus, if we suppose a pair of muscles, A and B, which under equal activity retain the lever on which they antagonistically operate in such a position that A is equal in length to B, and if we suppose that a new position be given to the lever such that A is longer than B, the neuro-muscular condition becomes altered so that A is more prone to be excited through the nervous arcs than is B. If I represent rightly in this way the principle arrived at by v. Uexküll, it will be seen that in some of the experiments mentioned in this Note and in my previous ones, the conditions resemble those in which v. Uexküll finds his principle at work.

A third process, qualified to play a part in linking together simpler reflexes so as to form from them reflex cycles of action, seems *successive spinal induction.* It appears especially fitted to combine the successive

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* 'Vergleichende Gehirnphysiologie,' Leipzig, 1899, p. 96.

† 'Zeitschrift f. Biologie,' vol. 44.

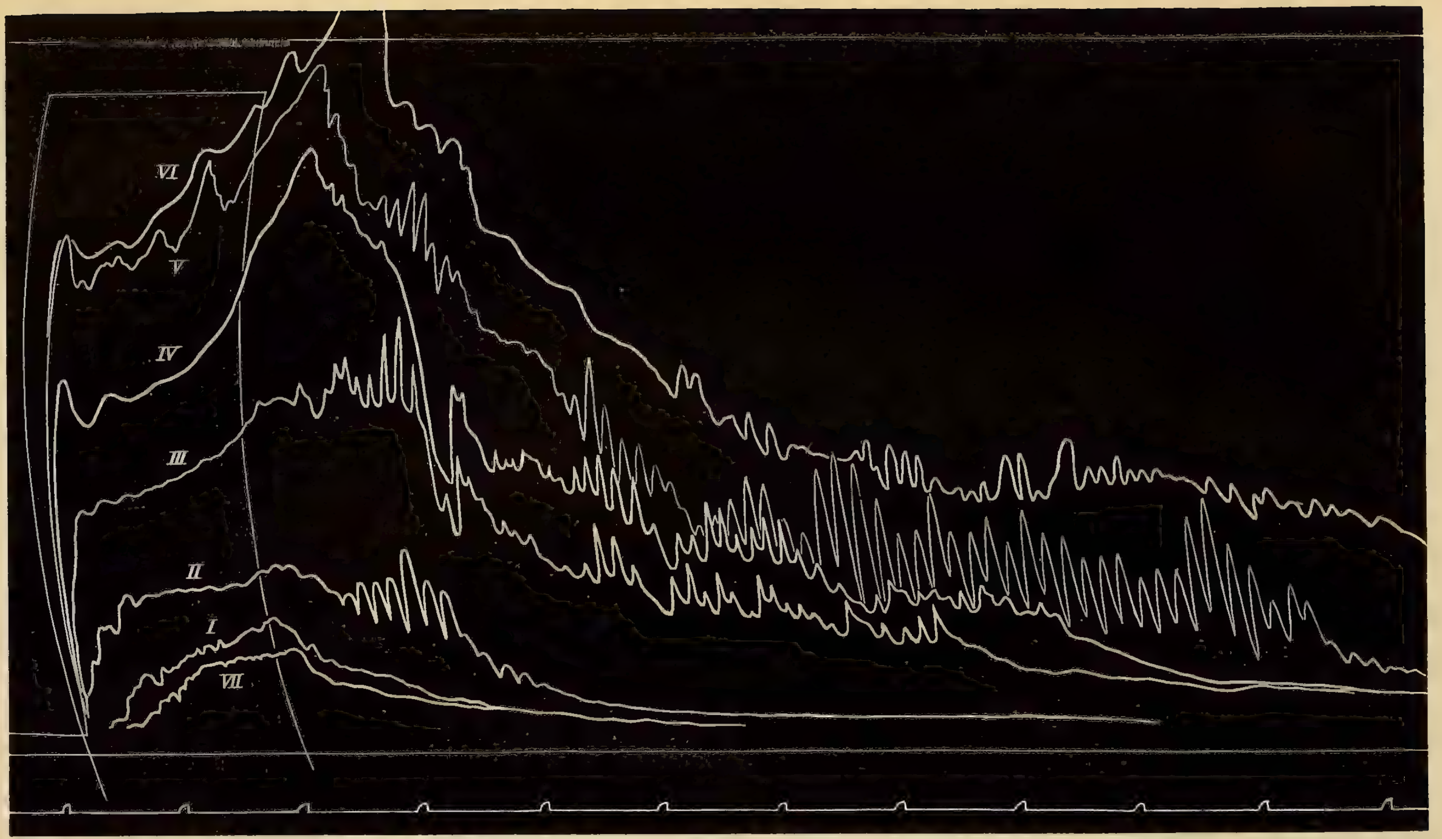

Fig. 8.—Flexion-reflex. Spinal dog. Grading of intensity of reflex with grading of intensity of stimulus. The stimulus for each reflex was 72 break induction-currents at the rate of 40 per second. This stimulus is registered above by an electro-magnet in the primary circuit of the stimulating apparatus. Abscissæ on the myograph curves show the moment of delivery and of cessation of stimulus in relation to those curves. The reflexes I, II, III, IV, V, VI, and VII were obtained in the order written at two-minute intervals.

| Reflex | Intensity of stimulus. | Measure of reflex. | Measure of after-discharge. |
|---|---|---|---|
| I | 350 | 94 | 51 |
| II | 475 | 200 | 119 |
| III | 690 | 666 | 509 |
| IV | 1100 | 913 | 692 |
| V | 1900 | 1272 | 964 |
| VI | 3000 | 1765 | 1452 |
| VII | 350 | 62 | 34 |

The clonus of the after-discharge is well seen in Curve V, which is here reproduced directly from the actual record, the other curves are transparency-tracings from the actual records obtained in the consecutive series of which V was the fifth member, and superposed on original tracing V for comparison and condensation of the record. Time below in seconds. From the same animal which yielded fig. 7.

opposite phases of such cyclic reflexes as I have termed "alternating,"* and shown to be particularly characteristic of the locomotor activity of the mammalian spinal cord. If a reflex, A, not only temporarily inhibits the action of an antagonistic reflex, B, but also as an immediately subsequent result induces in arc of B a phase of superactivity, the central organ is in that way predisposed for a second reflex opposite to A to occur in immediate succession to A itself. Such an effect seems proved by the observations in this and a preceding communication. A difficulty in applying it to the case of an ordinary alternating reflex, *e.g.*, the stepping reflex of the spinal dog, lies in the intensity and long duration of the reactions which I have employed in order to produce it experimentally. Such intensity and duration certainly do not occur in the course of the alternating reflexes as ordinarily observed. This, I think, does not exclude the likelihood that successive spinal induction is a factor which does contribute to the mechanism of alternating reflexes, although operating in smaller degree than as exemplified in the intenser examples obtained under experimental conditions and mentioned in this Note.

### *Addendum, March* 14, 1906.

Since concluding the above I have met with marked successive induction and rebound contraction following stimulation of the proximal end of one-half of the split *vasto-crureus* nerve when the stimulus has been quite brief and weak, *i.e.*, has not been detectible to the tongue-tip, and has lasted only from 1 to 2 seconds. Starting with the knee in semi-flexion, the stimulus has caused immediate relaxation of the *vasto-crureus* (inhibition), followed, on discontinuing the stimulus, by a rebound contraction, that has thrown the knee into full extension. This result has been obtained with mechanical, as well as electrical stimulation, of the nerve. It has also been obtained by stimulation of the central end of the whole *vasto-crureus* nerve after total severance of that nerve; the extension then obtained is due to extensors at hip and ankle, and guarantees that, in the rebound reflex, these latter extensors, as well as the extensors of the knee, are thrown into contraction.

The difficulty expressed in the concluding sentence above, of applying successive induction and rebound contraction to explain natural "alternating" reflexes, because of the lengthy and intense stimuli required to obtain them experimentally, is therefore greatly lessened. An instance of distinct rebound, induced by quite brief inhibition, is furnished in a figure illustrating the note† on innervation of antagonistic muscles next preceding

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* 'Phil. Trans.,' B, vol. 184, 1898.

† 'Roy. Soc. Proc.,' vol. B, 76, p. 277, April 17, 1905, 8th note, fig. 3.

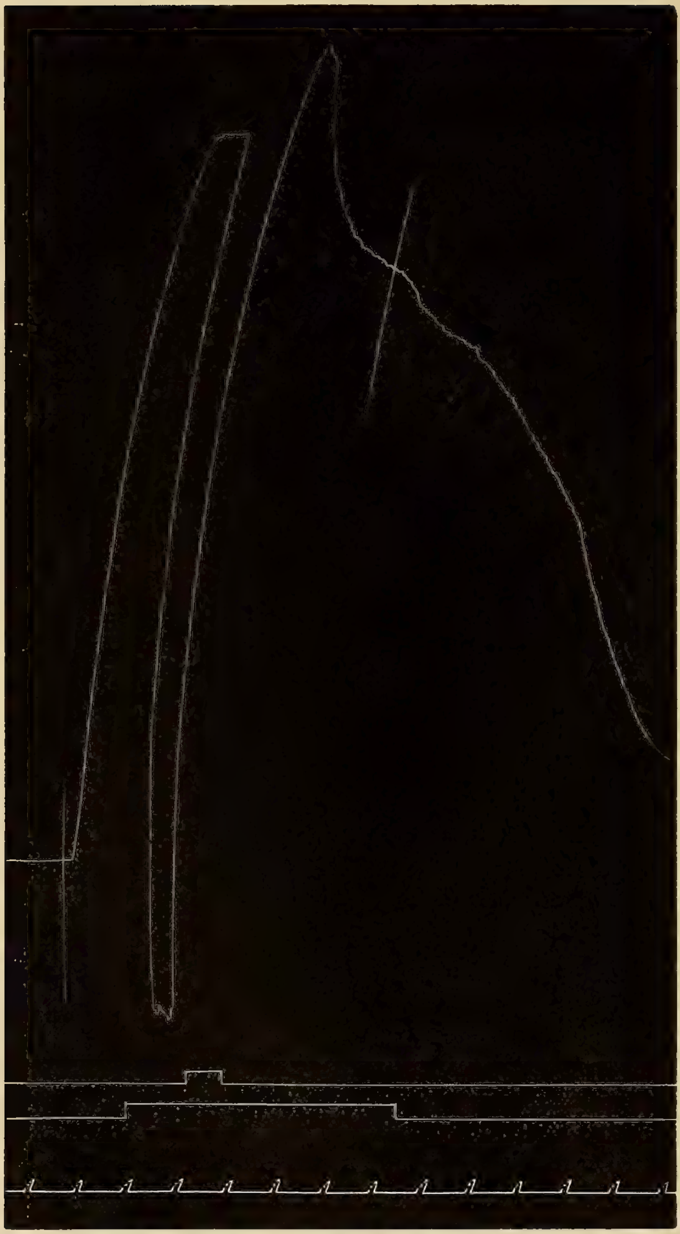

Fig. 9.—Myograph record of reflex contraction of extensor of knee interrupted by a reflex inhibition (relaxation). The reflex contraction was induced by stimulation (unipolar faradisation) of the skin of the opposite foot: this stimulation was applied during the time marked by the lower signal; its movements of commencement and ending are marked by abscissæ on the myogram. Towards the height of the reflex contraction a brief stimulation (unipolar faradisation) was applied to the skin of the foot homonymous with the knee extensor yielding the myogram: the duration of this inhibiting stimulus is marked by the upper signal. The knee extensor at outset was in some tonic contraction due to "decerebrate rigidity." The reflex inhibition relaxes this in addition to inhibiting the current reflex from the crossed foot. On return of the reflex subsequent to its inhibition there is distinct increase in the height of the reflex contraction as compared with the height of contraction immediately prior to the inhibition. Time is marked below in fifths of second.

the present. There an inhibition lasting only a fraction of a second is seen to induce a rebound of extensor contraction, which adds itself to a reflex concurrently in progress. Attention was not drawn to this feature in describing the tracing at the time, because I did not then appreciate it. The figure is here reproduced as fig. 9. The rebound is slight but distinct.

The successive induction, and its rebound contraction, are obtained after severance of the hamstring nerve. They are therefore not referable, as might, perhaps, be thought, to centripetal impulses generated in the hamstring muscles when contracting reflexly in response to stimulation of the afferent nerve of the extensor. The successive induction appears, therefore, directly, not indirectly, related to the stimulation of the afferent nerve of the extensor.

# *On the Existence of Cell Communications between Blastomeres.*

By Cresswell Shearer, Trinity College, Cambridge.

(Communicated by Adam Sedgwick, F.R.S. Received January 30,—Read February 15, 1906.)

[Plate 18.]

Several years ago in following the development of the larval excretory organs of the Serpulid *Eupomatus,* I had in the course of the investigation to section a large number of the gastrula stages of the egg of this worm. From time to time, in some of these sections I noticed delicate strands of protoplasm traversing the segmentation cavity or portions of this space, connecting one cell with another, or one blastomere with its neighbour. These connections were similar in many respects to the "filose" strands described by Andrews (1), which he has observed joining the blastomeres, in a number of Metazoan eggs during life. I also noticed they were sometimes present in advanced stages where their filose character was somewhat different from their appearance at an earlier date. I paid them little attention at first, considering them the result of the action of the fixing reagent in coagulating the albuminous fluid of the blastocoel, or what was still more probable, to the imperfect penetration of the reagent, and the consequent necrosis and disintegration of the protoplasm in the interior of the egg. Meeting with them again and again in the course of the work, and under conditions that seemed to show they were not due to either of these causes, I was led to make a few experiments to determine if possible their nature and significance.

Their presence, I found, was well marked, in eggs preserved with any of the good fixatives, especially those that act rapidly, such as the Osmic acid mixtures, and Corrosive-acetic. The action of these is sufficiently different to preclude the strands being therefore of the nature of ordinary coagulation artifacts. On the other hand, it was impossible to ascribe them to the breaking up of the protoplasm and imperfect fixation, as in many of the eggs in which they were present all the finer histological details were well preserved, and in some cases the various phases of mitosis plainly shown.

Their observation on the living egg I found difficult on account of their transparent nature during this state, and also on account of the difficulty of examining the interior of the segmentation cavity satisfactorily in the whole egg under the high power of the microscope. By placing a light cover glass supported at its four corners by means of wax feet, over a few eggs in a drop of sea water on a slide, it is possible to hold the eggs in any position, and study them if necessary for hours under an oil immersion lens. By pressing down the cover glass, the eggs can be flattened slightly without causing any serious injury or displacement of the cells. It is then possible to closely examine the interior of the blastocoel. In preparations of this kind, delicate strands can be seen in some cases connecting the blastomeres, their appearance being much the same as in fixed material with the exception that they are less granular and finer in appearance. Unfortunately this method of examination may be held to cause their formation.

The fixation of the sections from which the accompanying drawings (Plate 18) were made was in all cases good, Flemming, Hermann's fluid, or Corrosive-acetic being used.* The sections were cut in the ordinary way in paraffin; very dilute paracarmine being used as a stain.

Fig. 1, is taken from a section of an egg of *Eupomatus*, three hours after fertilisation, and fixed with Flemming's strong solution. A fine strand of protoplasm is seen connecting one blastomere with another across the segmentation cavity. The granules of the protoplasm of one cell, are traceable without break in continuity throughout the course of this strand, into that of the other. By examining the consecutive sections through this egg, it can be readily determined that this strand does not represent a ridge on the wall of the cavity so cut as to appear as a strand in the section. It is quite plainly in the middle of the cavity, running freely from one wall to the other. In this and the subsequent sections the darkly staining egg membrane is seen surrounding the egg, and is remarkable in *Eupomatus* for its thickness, remaining about the egg till a relatively late stage in development.

Fig. 2, is also taken from a *Eupomatus* egg, some four hours after fertilisation fixed in Flemming's solution. Here several strands are seen irregularly crossing the cavity, one strand being shown cut across in the section. In the cavity a number of granules are seen.

Fig. 3, is from an egg somewhat similar to that from which the last section is taken. In one of the cells a conspicuous mitotic spindle is shown cut across. The large cells (marked *en* in the figure) on the lower side of the section subsequently become the entomeres, while the dorsal cells with which they connect, become the "apical rosette" cells, which are plainly

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* It is worthy of note that Wilson (17), in comparing the appearance of living with fixed protoplasm, comes to the conclusion that its treatment with suitable reagents "does not materially distort or pervert the normal structure, but gives on the whole a remarkably faithful picture of the structure existing in life," (p. 5).

present at a slightly later stage. In living material, strands such as these are seen to arise as the gradual separation of the cells takes place to form the segmentation cavity; so in some cases they would seem not to be new formations, but the stretching of former connections. For instance, the strand in fig. 1, can be sometimes seen to form as the cells are separated by growth, by the drawing out of their former point of contact, their ends being in touch with one another at an earlier date.

Fig. 4, is also taken from a section of a *Eupomatus* egg, but this time fixed with Hermann's fluid. In this case a distinct ridge is cut across, but no cell boundary can be distinguished. The protoplasm granules are continuous without interruption from one cell to the other.

Fig. 5, is a section of a partially-formed gastrula of *Eupomatus*. Here one of the endoderm cells has joined two of the ectoderm cells. But at the point of contact no cell wall can be distinguished. The upper of the two ectoderm cells making the connection, probably belongs to the "apical rosette cells," and the entomere with which it forms the connection is possibly the cell shown in fig. 3 (marked *en*) at a later stage. This connection may have remained unbroken from the first.

Fig. 6, is a gastrula of *Eupomatus* fixed with corrosive and acetic. The ectoderm and endoderm are joined by strands which are continuous with cells in each layer. These strands may represent mesenchyme cells, but at this stage they would seem to be joined alike to cells in both germ layers.

Fig. 7, is a section similar to some of the first. The strands in this case are decidedly filose in character.

Fig. 8, is a section of a gastrula of *Polygordius* fixed with Corrosive-acetic. A large entomere is seen continuous with a conspicuous cell of the ectoderm.

Numerous other examples could be given, but it is unnecessary to add to their number, as in all cases the strands are of quite the same nature. There can hardly be any doubt that they are similar if not identical with the filose filaments described by Andrews (1), as already mentioned, and which he expressly states sometimes "varied from fine filaments to broad bridges." He has observed them on the living eggs of a number of Echinoderms, Annelids, Molluscs, and Nemertines, and traces of their presence in preserved eggs of *Amphioxus* and Amphibia. He says, "the most delicate filose displays were seen near the polar bodies during the first and second cleavages. The egg put forth fine protoplasmic threads that branched and reached up towards the second polar body, and the filose phenomena in it led to the assumption that it was a flowing mass of protoplasm" (p. 5). According to Andrews, who believes they "furnish a medium for co-ordinating the activities of the parts of the embryo," they form as thread-like pseudopodia of the hyaloplasm similar to the way Radiolaria and Foraminifera throw out this layer in the formation of their long and delicate processes. In some cases and possibly in all cases except where they form as already mentioned as the drawing out of established connections, my own observations also lead me to believe they are essentially of the nature of pseudopodia.

In the living egg they are plainly seen to possess pseudopodium-like movements, varying in shape and size from time to time. As in the pseudopodia of *Gromia oviformis*, the classical object of study, the granules of one thread are plainly seen to pass into another where they touch or overlap, so the granules in the protoplasm of the blastomeres can be traced along these strands from one cell to the other. Frequently the strands have a beaded appearance not unlike the appearance of the pseudopodia in *Orbitolites* after stimulation.

In the Annelid *Podarke*, the egg of which is about the same size as that of *Eupomatus*, and has a form of cleavage as far as I can determine very similar step for step, it is interesting to note that Treadwell (13), in speaking of the 40-cell stage, says " strands of protoplasm can be plainly seen reaching across the cleavage cavity " and " protoplasmic connections do exist between blastomeres of relatively late cleavage stages " (p. 410). His text-figures 3 and 4, show two strands crossing the segmentation cavity from the apical rosette cells and joining two of the entomeres, here, as Treadwell suggests, possibly the contact between them from an early stage has never been broken.

During segmentation, the egg of *Eupomatus* seems to have alternate periods of rest and rapid division. During the periods of rest the cell boundaries seem to fade out, and the nuclei become large and peculiarly opaque. Cutting sections of eggs in one of these resting periods, no matter how well fixed, it is next to impossible to distinguish cell outlines, and in the early stages the egg looks not unlike a multinucleated mass of protoplasm. I have noticed that the first signs of the assumption of the period of division are shown, by the reappearance of the cell outlines, when the cells seem drawn apart from one another to some extent. It is at this time that the filaments are most obvious and numerous. When the period of active division has fully set in they are seldom seen.*

Frommann (2) years ago drew attention to fine lines running between the blastomeres of *Strongylocentrotus* eggs, which had been preserved first in weak and then in strong alcohol. These threads seem to be outside the cell membrane as far as one can judge from his figs. 14 and 16. They look not unlike coagulation artifacts, and it is doubtful if they are really protoplasmic in nature.

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* These periods of rest and active division are used as applying to the appearance presented in preserved material. I am not certain that they are to be distinguished during life. As far as I have observed, growth is steady, although material preserved at intervals shows these alternating periods of what I have termed rest and division.

Hammer (4) has recently described bridges of protoplasm that connect blastomeres at the ends of their planes of contact. These are most obvious in the two-cell stage, a delicate bridge joins the blastomeres at each end of the cleavage plane. I have frequently observed these connections on the eggs of *Pomatoceros* where they are well marked. But the best examples are furnished by the eggs of *Phoronis.* In the two-cell stage they are plainly evident, and on the living egg one can readily trace with the high power of the microscope, protoplasmic granules continuously from one cell to the other through these bridges. Klaatsch (6) has described somewhat similar connections, on the egg of *Amphioxus,* though he represents bridges connecting the cells along the course of the cleavage planes as well. In the gastrula of *Amphioxus* he shows all the cells connected by delicate lines. They however look not unlike the result of shrinkage during preservation.

Both Hammer and Klaatsch discuss the possible influence of these cell communications in co-ordinating the activities of the individual cells. The results of experimental embryology of the last decade amply show that such influence must exist, and that it is impossible to hold that in the growing organism the cell lives an isolated existence. Wilson (16) in speaking of the results of his work on the mechanical separation of the blastomeres of *Amphioxus,* states, "There must be a structural continuity from cell to cell that is the medium of co-ordination," and that it is this that "is broken by mechanical displacements of the blastomeres."

Townsend (12), has shown how in plants very delicate strands can transmit the influence of the nucleus from cell to cell. In the plasmolysed root-hairs of *Marchantia* the protoplasm is broken up into balls, some of which only are nucleated, the nucleated portions alone are capable of utilising the starch they contain and of surrounding themselves with a new cell wall. But some of the non-nucleated portions are sometimes connected with a nucleate portion by long delicate strands or threads, they then, as the result of this connection with the nucleus, elaborate cell walls; if this strand is however broken they lose this power and soon die. While it is well known that the nucleus plays the essential part in the formation of new products chemical or morphological of the cytoplasm, it is remarkable to find this power capable of being transmitted from one mass of protoplasm to another, or as in the case of the leaf-hairs of *Cucurbita,* from cell to cell by means of such delicate threads. It is highly probable that the strands described here between blastomeres can play an important part in a similar manner.

In nearly all plant tissues, the cell walls are traversed by delicate strands or intercellular bridges of protoplasm. The work of Gardiner (3), Kienitz-Gerloff (5) and Meyer (7), has conclusively proved among plants how wide-spread and universal is their distribution. It is almost possible to speak therefore of plants as multinucleated masses of protoplasm or *Syncytia*. Meyer (7), as the result of his studies on *Volvox*, comes to the conclusion that "Plasmaverbindungen zwischen allen Zellen eines jeden Individuum dadurch vorkommen, dass das thierische und pflanzliche Individuum charakterisirt ist, dass es eine einheitliche Cytoplasmamasse besitzt, dabei eine einkernige Zelle, eine vielkernige Zelle oder ein System von Zellen sein kann, deren Cytoplasma ein zusammenhängendes Ganzes bildet," p. 212.

Among animals, undoubted cases of true syncytia occur not infrequently in the centrolecithal eggs of Arthropods. Here the nucleus takes up a central position, dividing into two, four, eight, and sixteen or more cells, while the egg as a whole has remained unsegmented. The nuclei make their way to the surface of the egg there taking up their respective positions; at a later stage only after this has taken place, does the cytoplasm split up into as many cells as there are nuclei.

In *Peripatus*, according to Sedgwick (11) the cleavage takes place as in centrolecithal eggs, but the segmentation nucleus divides at the periphery instead of the centre of the egg. The first division separates the opaque area into two portions that lie closely applied to one another. A careful examination, however, shows "that the furrow has not completely separated the two segments from each other, but that they are connected by strands of protoplasm forming a loose network between them, the embryo at the gastrula stage, and in all the earlier stages of development, is a syncytium," p. 24. Sedgwick holds that even in later stages, and also in Vertebrates, the entire body is one continuous syncytium. "The primitive streak, the walls of the cœlom, and the neural crest, and parts of the ectoderm, are growing points where nuclei, not cells are produced." He cites in particular the case of the third nerve in Selachians, which he claims along with other nerves is differentiated as a portion of a continuous reticulum. Amongst adult epithelial and connective tissue cells connections or bridges were early described by the old school of histologists as Max Schultze (1864), Bizzozero (1872), Ranvier, Flemming (1876), Heitzmann (1883) and recently Schüberg (9) has added a long contribution to the subject in which many of these early results are confirmed.

Against either Meyer's or Sedgwick's theory, cases can be cited of cells in the body, as the blood cells, the mesenchyme of many Invertebrates, which are quite free; but perhaps the force of these and similar facts is removed when we consider that fixed and specialised tissue cells, such as the squamous epithelium of the cornea, are capable of assuming the character of leucocytes and wandering free as the response to injury. Certainly in view of all the evidence that has been gradually accumulating, our conception of the cell theory needs remodelling on some such lines as Meyer has laid down. This has clearly been pointed out by Sedgwick (10) and Whitman (14). In the words of Sachs (8) cells are "merely one of the numerous expressions of the formative forces which reside in all matter."

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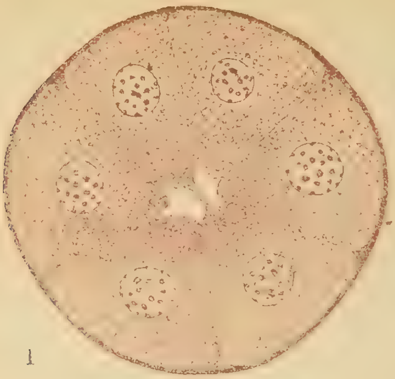

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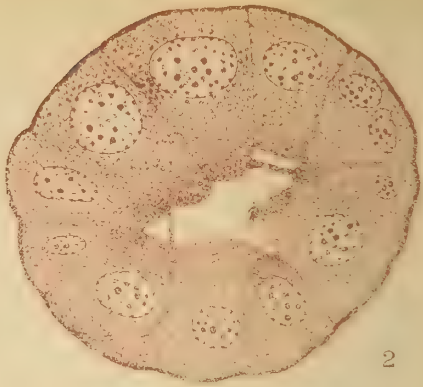

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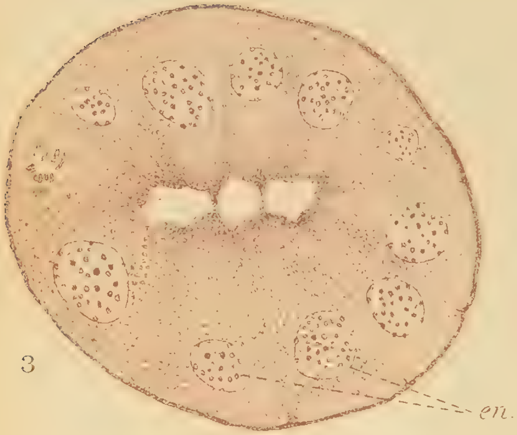

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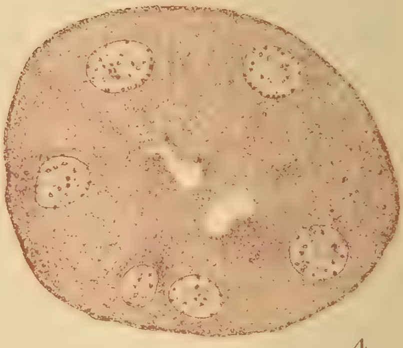

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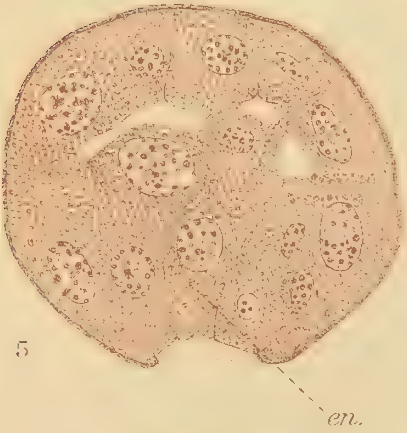

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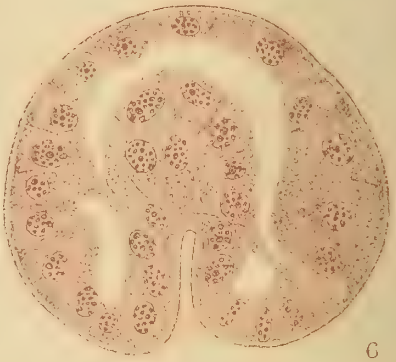

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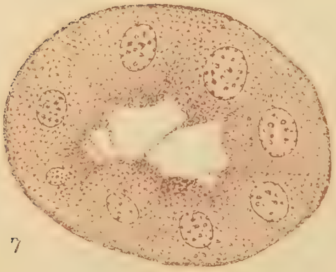

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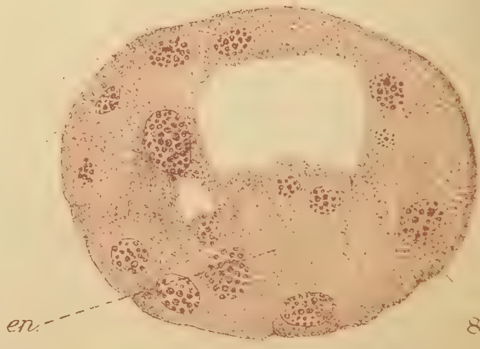

8

Shearer del.

West, Newman chr.

1 - 7 EUPOMATUS 8 POLYGORDIUS

## DESCRIPTION OF PLATE.

All the figures have been drawn under an oil immersion, 2 mm. obj. No. 6.

Figs. 1, 2, 3.—Sections of *Eupomatus* egg. Fixed in Flemming. × 600.
„ 4.—Section of *Eupomatus* egg. Fixed in Hermann solution. × 600.
„ 5.—Section of gastrula of *Eupomatus*. Fixed in Flemming. × 600.
„ 6.—Section of a gastrula of *Eupomatus*. Fixed in Corrosive and acetic. × 600.
„ 7.—Section of *Eupomatus* egg. Fixed in Flemming. × 600.
„ 8.—Section of a gastrula of *Polygordius*. Fixed in Corrosive and acetic. × 600.

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# *An Experimental Enquiry into the Factors which Determine the Growth and Activity of the Mammary Glands.*[*]

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(From the Physiology Laboratory, University College, London.)

[Plate 19.]

The correlation between the mammary glands and the other organs concerned in generation presents perhaps the most striking example of the interdependence of the growth and activities of different organs of the body. Although the manner in which this correlation is brought about has been the subject of speculation for many years, especially among medical men, it is only quite recently that any attempt has been made to apply experimental methods to its explanation.

In the case of the mammary glands we have organs which are present in both sexes, and at birth are in the same immature condition. In both sexes there is frequently, during the few days after birth, an enlargement of the glands and an actual secretion of fluid, which is known as "witch's milk." This state of activity disappears at the end of the first or second week, and till puberty the glands remain in the same undeveloped condition. At this period in Man the first difference appears between the mammary glands of the two sexes, a rapid growth taking place in the female, and accompanying the commencement of the ovarian functions.

During adult life there is apparently at each œstral period a slight and temporary enlargement of the gland, and during the whole of adult life the glands remain in the female at the same state of development unless pregnancy occurs. The commencement of pregnancy acts as an impetus to a further great development of the gland substance, a development which, as we shall see shortly, differs in some respects from that which gave rise to the growth of the gland at puberty. This hypertrophy continues throughout the whole of pregnancy, being more rapid as pregnancy advances.

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[*] In this investigation the operations and inoculations were carried out by E. H. Starling, and the preparation of the extracts and the microscopic examination of the glands by J. Lane-Claypon.

With cessation of pregnancy, whether abnormally or at the normal term, the growth of the glands at once comes to a stop, and two or three days after this cessation the activity which previously was spent on growth is now applied to the secretion of milk, a secretion which, if the gland is emptied periodically, lasts for many months.

This whole cycle of changes can be prevented by the removal of the sexual glands, namely, the ovaries, and these organs must therefore be regarded as primarily responsible for the growth of the mammary glands. We shall have to consider later on whether they are the immediate source of the impulses which induce the growth of the gland during pregnancy and its secretory activity after parturition. We must conclude, however, that there is some impulse or impulses arising in the ovaries, uterus, or product of conception, which exerts a direct influence on the mammary glands, and is responsible for their hypertrophy.

What is the nature of this connection? The well-known nervous connections between the mammary glands and the uterus, instanced by the occurrence of uterine contractions on stimulation of the nipples, has been taken to indicate that the impulses which cause growth of the glands are also nervous in character. Although this view is still sustained by certain physiologists, *e.g.*, K. Basch,* there are many facts, experimental as well as chemical, which place such an explanation out of court. Thus in a goat Eckhard† cut all the nerves to the mammary glands without appreciably affecting the yield of milk from the denervated gland. Ribbert‡ transplanted in the guinea-pig one of the two mammary glands to the neighbourhood of the ear, thus dividing all its nervous connections. In a subsequent pregnancy this gland enlarged as usual, and milk could be obtained from it at the end of pregnancy.

Still more definite evidence is afforded by the well-known case in which Goltz and Ewald§ removed the whole of the lumbo-sacral cord in a pregnant bitch, thus severing all possible nervous connections between the pelvic organs and the mammary glands. This dog gave birth to puppies at full term. Pregnancy was attended with the normal hypertrophy of the mammary glands, and the bitch successfully suckled one of the puppies. A similar case has been recorded by Routh.*

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* 'v. Ergebnisse der Physiologie,' vol. 2, 1, p. 325, *et seq.*, 1903; also Bibliography.

† 'Eckhard's Beiträge,' vol. 1, p. 18.

‡ 'Archiv f. Entwickelungsmechanik,' 1898.

§ 'Pflüger's Archiv,' vol. 63, p. 385.

A woman when seven months pregnant was the subject of fracture of the spine at the sixth dorsal vertebra, completely crushing the spinal cord at this level. Parturition occurred two months later, and the woman was able to suckle the child normally.

Moreover we have experimental evidence that the correlating mechanism between the different parts of the generative system is not nervous. Thus Knauer† showed that, whereas extirpation of both ovaries puts an end to the periodical changes in the uterus which are responsible for the phenomena of "heat," both ovaries can be transplanted, thus dividing all their nervous connections, without undergoing complete atrophy and without abolishing the phenomena of "heat." We must conclude therefore that the connecting link in all these cases is chemical rather than nervous. It remains for us to determine the origin of the exciting substance or substances, as well as their mode of action.

In the case of the mammary glands we have really two questions to decide. We have to determine why the secretion of milk appears only at the end or after the termination of pregnancy, and in the second place the origin of the stimulus which, during pregnancy, is responsible for the hypertrophy of the mammary glands.

### *The Causation of the Secretion of Milk after Parturition.*

In Man the secretion of milk commences two to three days after parturition. During the greater part of pregnancy a fluid can be expressed from the breasts, and this fluid can be obtained in greater quantity after birth; it is not true milk, but a watery fluid rich in proteids, which is called colostrum. In certain recorded cases where pregnant women have suckled during pregnancy, the fluid that has been obtained has also not been true milk, but of the nature of colostrum. In some animals, such as the rabbit, true milk can be obtained from the glands during the last two days of pregnancy. In each case, however, the factor which sets the secretory process going must be the same, and the question at once arises whether the secretion is due to a change in the metabolism of the uterus or ovaries, or to the absorption from the involuting uterus of products which may act as a special stimulus to the hypertrophied gland.

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* 'Obstetrical Society of London Trans.,' vol. 39, 1897.

† 'Archiv f. Gynäkol.,' p. 322, 1900.

Observations on Man show that the secretion of true milk cannot be due to a stimulus received by the gland at the time of conception, since the secretion occurs whenever pregnancy comes to an end, whether at the third month or at the ninth. A good account of the clinical evidence bearing on this point has been given by Halban.* This observer points out that when premature death of the fœtus occurs without its expulsion, as in cases of extra-uterine fœtation, the breasts swell two or three days after the death may be presumed to have taken place, and a true secretion of milk may be obtained.

The same thing occurs when the death of the fœtus happens *in utero*, but the result is not invariable. Thus Halban mentions certain cases in which the secretion of milk began two or three days after the presumed death of the child, but he also gives details of other cases in which the secretion occurred only after the expulsion of the dead fœtus. Halban is inclined to ascribe the difference between these two classes of cases to differences in the placentæ, and imagines that the appearance of milk in the breasts is determined, not by the removal or the death of the living fœtus, but by the removal or death of the placenta.

The hypothesis that some special stimulating substance is formed in the involuting uterus or in the ovaries after parturition seems to be negatived by certain cases in which Porro's operation was performed without interfering with the subsequent onset of lactation. In order, however, to decide this point we have carried out a number of observations on pregnant rabbits. In these the whole uterus, uterine appendages, and ovaries, were removed at different stages of pregnancy. In the rabbit pregnancy lasts, on the average, 28 to 30 days. If in a primiparous rabbit Porro's operation be performed at the 10th day or at any day before the 14th, the development of the mammary gland at once ceases and gives place to retrogression. No milk, however, appears in the gland, and at the end of a couple of months the gland is little removed from that of a virgin animal. If, on the other hand, the operation be carried out on animals after the 14th day of pregnancy, within two days after the operation milk can be expressed from the nipples, showing that here, as in Man, the effective factor in determining the process of lactation is not the absorption of substances from ovaries or involuting uterus, but the *removal* of certain stimuli which normally proceed from the organs or fœtus of the pregnant animal.

The most obvious explanation of these results is that lactation is due to the removal of the stimulus which during pregnancy occasions the hypertrophy of the mammary gland. Hildebrandt† has suggested that during pregnancy an impulse is exerted by the developing ovum on the mammary glands which acts as a stimulus to growth, and at the same time protects the cells of the gland from those autolytic disintegrative processes which occur to a large extent in the secreting gland. That the act of secretion can be ascribed to autolytic processes of the gland seems highly improbable. We have no evidence that the autolysis of the gland cells would give rise to the specific constituents which characterise milk, but Hildebrandt's idea of an inhibitory substance which excites hypertrophy of the gland, and whose removal leads to secretion, is a valuable one.

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* 'Archiv f. Gynäkol.,' vol. 72, Heft 2, 1905.

† 'Hofmeister's Beiträge,' vol. 5, p. 413, 1904.

Our whole idea of inhibition involves the stopping of the normal dissimilative processes in a tissue and the augmentation of the assimilative, so that the continued reception of inhibitory impulses must lead to an actual increase in the substance of the cells, with resulting division and growth of the gland. But, as Hering has pointed out, the more this process of assimilation is carried on, the greater is the tendency to autonomous dissimilation, and the removal at the end of pregnancy of inhibitory impulses, or the attainment towards the end of pregnancy of a maximum degree of hypertrophy, must result in an autonomous dissimilation, *i.e.*, activity of the tissue. The normal activity of the mammary gland is secretion, and it is entirely in accordance with the accepted principles of physiology that the stimulus, which during pregnancy gave rise to growth, should by its removal at the end of pregnancy be responsible for the act of lactation.

We may conclude then that lactation is due not to excitation of the gland by special substances produced in the other generative organs, but to the removal of the stimulus which during pregnancy was responsible for the growth of the gland.

### *The Origin of the Substance or Substances by which the Hypertrophy of the Mammary Glands is determined.*

We have seen reason for regarding the development of the mammary glands, which occurs in the female at puberty, as being due to the production of some substance in the ovaries which must reach these glands by way of the blood. It is probable that the slight increase in size of these glands at each œstral period is also determined by the greater activity of the ovaries at these periods, an activity which has been shown by Marshall* to be probably responsible for the changes which occur in the uterus. These changes are, however, insignificant as compared with the enormous hyperplasia which is associated with pregnancy. The question arises whether the greater growth during pregnancy is to be ascribed to an increased production of the specific chemical excitant by the ovaries, or to the changed metabolism of the uterus during pregnancy, or whether the new products of conception, namely, the fœtus and placenta, represent the seats for the manufacture of the mammary hormone.

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* 'Phil. Trans.,' B, vol. 198, 1905.

There is a large amount of clinical evidence which enables us to decide with some probability, though without certainty, between these various alternatives. Conception is followed by a great increase in the size of the ovaries, chiefly in consequence of the formation of the *corpus luteum.* This growth has been associated by Born, Frænkel,* and Marshall† with the production of a specific excitant for the uterine mucous membrane which determines the growth of this membrane and the attachment of the embryo. These observers have shown that extirpation of both ovaries at an early period in pregnancy, or even destruction of the *corpora lutea* by means of the cautery, causes the cessation of the pregnancy. We ourselves have found that extirpation of both ovaries in the rabbit if carried out before the 15th day is apt to cause abortion. Later on in pregnancy oophorectomy can be performed without interfering with the course of the pregnancy.

The growth of the ovaries is, however, chiefly marked in the first third or half of pregnancy. After this time the *corpora lutea* begin to diminish in size, and with them the whole ovaries. On the other hand, the growth of the mammary gland, although marked almost immediately after impregnation, becomes more and more rapid with the advance in pregnancy, and the growth during the last half of pregnancy is many times greater than that during the first half. Moreover, numberless instances in man show that extirpation of both ovaries, as in double ovariotomy, does not necessarily interfere with the course of pregnancy or with the growth of the mammary glands. Labour may come on at the usual time, and be followed by normal lactation.

The fact that the mammary glands undergo normal hypertrophy in extra-uterine fœtation, where the growth of the uterus is only a small fraction of that occurring under normal circumstances, seems to point to the products of conception, *i.e.*, either the fœtus or placenta, or both, as the seats of origin of the mammary hormone. This conclusion is borne out by the fact that in extra-uterine fœtation, if the death of the fœtus occurs, this event is followed within a few days by the appearance of milk in the hypertrophied gland. The same result occurs when the death of the fœtus happens *in utero* some time before its expulsion. On the other hand, as mentioned in the earlier part of this paper, cases have been recorded in which the death of the fœtus probably occurred a considerable time, a week or more, previous to its expulsion, and yet the appearance of milk in the gland has only taken place two or three days after the actual labour. Halban concludes, though, as it seems to us, on insufficient grounds, that the difference between the two sets of cases depends on whether the death of the placenta accompanies that of the fœtus, and locates the seat of production of the specific hormone not in the fœtus, but in the chorionic villi and placenta.

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* 'Arch. f. Gyn.,' vol. 68, 1903.

† *Loc. cit.*

It is evident that no satisfactory solution of this question can be arrived at except by experiment. If any of the organs we have mentioned is the seat of production of a hormone which determines the growth of the mammary gland, it should be possible to obtain this hormone by extraction of the organ, and by its injection into a virgin animal produce the changes in the mammary glands which are characteristic of pregnancy. It is on these lines that we have proceeded in the present research, and have chosen rabbits as subjects of experiment since they can be obtained in large numbers, are easily handled, and breed fairly well in confinement. Before describing the experiments themselves we must say a few words as to the normal course of development of the mammary gland in this animal during pregnancy.

### *The Natural Growth of the Mammary Glands.*

In a virgin rabbit of eight months to a year old, the average age of the rabbits used in our experiments, it is difficult with the naked eye to see any trace of the mammary gland in the tissue lying under the nipples. In order to bring the gland into view we have removed the skin with the subcutaneous tissue, dissected the latter away from the skin, stretched it on a ring of cork, and then, after hardening, stained it in a weak solution of hæmatoxylin. After staining, the subcutaneous muscle fibres were dissected away, leaving only connective tissue, with any mammary gland tissue which might be present, surrounding the nipple. In the spread-out specimen it is possible to see in the immediate neighbourhood of the nipple the ducts which form the mammary gland. As a rule they are limited to an area not more than 1 cm. broad. Plate 19, fig. 1, shows the largest extent of mammary gland we have ever observed in a virgin rabbit of this age. On section the gland is found to consist entirely of ducts, which are lined with a single layer of flattened epithelial cells, and terminate blindly. In no case is there any trace of the alveoli which are so characteristic of the fully-formed gland.

With the occurrence of conception a marked change begins in the gland. Four or five days after conception, when it is still impossible with the naked eye to discover any embryos in the swollen uterine horns, on reflecting the skin from the abdomen each mammary gland appears as a circular pink area, about 2 to 3 cm. in diameter, surrounding the situation of each nipple (fig. 2). On section the gland is seen to consist still merely of ducts. These however are in an active state of proliferation, throwing out tree-like branches towards the periphery. The epithelial lining of the ducts is two or three cells thick; the cells are much more swollen than in the virgin gland, and numerous mitotic figures are seen in the epithelium. This growth proceeds rapidly, so that about the ninth day, on reflecting the skin from the abdomen, the whole is found to be covered with a thin layer of gland tissue. The margins of the glands are now almost contiguous, and the glands vary in diameter from 5 to 8 cm. (fig. 3). The appearance of the branching ducts in the stained specimen is also somewhat different, the ducts presenting, especially towards the periphery of the gland, cauliflower-like excrescences from their margins. The extreme periphery of the gland is generally somewhat thicker than the rest of the gland. On section it is found, especially at the periphery, that the formation of alveoli is commencing. This formation of alveoli proceeds henceforth rapidly, together with a continued growth of the ducts. At the twenty-fifth day the whole surface of the abdomen is covered with a layer of gland tissue, which may be 0·5 cm. thick, and on section is found to be formed for the greater part of alveoli. About this date, too, fat globules are forming in the cells of the alveoli. In the rabbit pregnancy lasts about 30 days. During the greater part of pregnancy, from the ninth day to the twenty-fifth, a watery fluid can be squeezed from the nipples. During the last two or three days of pregnancy this fluid becomes milky in character, so that immediately after parturition the mammary glands are already full of milk.

## THE ARTIFICIAL PRODUCTION OF GROWTH IN THE MAMMARY GLANDS.

We may now proceed to an account of the experiments in which we tried to reproduce artificially some of the remarkable changes which are normally determined by pregnancy. It was evident to us, before we began our researches, that it would be difficult, if not impossible, to present any stimulus to the mammary glands which would be as effective as the normal one. For, wherever the mammary hormone is manufactured, the manufacture must be assumed to proceed continuously. There is therefore a constant leakage of the active substance into the blood, and it is probable that the amount of this substance produced increases with the duration of pregnancy. At no time will the mammary gland be set free from the influence of this specific stimulus. On the other hand, however, we might prepare our extracts of the tissues, we could not expect to get more than the amount residual in the tissue and caught, so to speak, in its progress through the placenta into the maternal blood-vessels. This amount we might inject into our rabbits, but it would probably be taken up and absorbed into the circulation of the rabbit long before we were ready for our next injection, so that, whereas under normal circumstances the mammary glands are being continuously stimulated to hyperplasia, we could not expect to do more than give these glands a series of small shoves in the same direction.

### *Methods of Experiment.*

Our object in the present research was to attempt to imitate the processes of pregnancy, by the injection of fluid extracts containing the soluble constituents of the various tissues, ovaries, uterus, placenta, or fœtus into a virgin rabbit. The extracts were prepared in various ways. In some of our earlier experiments an emulsion of the tissue in normal salt solution, prepared with aseptic precautions, was injected subcutaneously. This method limited us to the use of only small portions of tissue. Since the other hormones with which we are acquainted, such as secretin or adrenalin, are soluble and diffusible substances, in later experiments we used a much greater mass of tissue for the preparation of fluid extracts, which were made sterile and freed from solid particles by passage through a Berkefeld filter. In all cases the tissues were ground up thoroughly with sand. The resulting paste was then in some cases mixed with normal salt solution, allowed to stand for two or three hours, and centrifuged. The supernatant fluid was passed through a Berkefeld filter, and the resulting clear filtrate used for injection. In other cases the ground-up tissues were mixed with Kieselguhr, and the juices expressed in a Büchner press; the press-juice thus obtained was passed through a Berkefeld filter before injection. The injections—which were naturally made with aseptic precautions—were in the early experiments subcutaneous. As, however, we increased the bulk of the injection, we adopted the plan of injecting into the peritoneal cavity. A further advantage of injecting into the peritoneal cavity is that one not only obtains quicker absorption, but is free from the disadvantage attendant on subcutaneous injection, namely that the fluid introduced under the skin of the back tends to flow down to the subcutaneous tissue in the abdomen, so that the mammary glands are bathed in an aseptic fluid containing proteids, which might in itself serve as a stimulus to proliferation.

### *Experiments on Injection of Tissue Extracts.*

Our experiments can be divided into the following groups according to the organs injected, namely injection of ovaries, of uterine wall or mucous membrane, of placentæ, of placentæ plus uterus, of fœtus, together with placenta and membranes, or of the fœtus alone. Finally, we made certain control experiments in which the animals were injected either with emulsion of liver or with blood serum obtained from normal animals.

### I.—*Ovaries.*

*Experiment* 1. April 22, 1904.—Two ovaries from rabbit 15 days pregnant were implanted into peritoneum of second non-pregnant rabbit.

May 3.—Rabbit killed. No change in mammary glands; the implanted ovaries were necrosed and vascularised; uterus and vulva congested. On microscopic examination there was no proliferation of mammary gland. The uterine mucous membrane was typical of "heat."

The rabbit from which the ovaries were taken aborted immediately after the operation.

*Experiment* 2. May 31, 1904.—A rabbit which had been in the laboratory since January was injected with chloroform water extract of six ovaries from three rabbits, each about the fifteenth day of pregnancy. The rabbit received three injections in the course of nine days.

It was killed on June 13, four days after the last injection. No changes were found in the mammary glands, and there were no changes in the uterus.

*Experiment* 3. June 6, 1904.—A virgin rabbit was injected on six occasions during 19 days with an aseptic emulsion of 10 ovaries from rabbits between 11 and 14 days pregnant.

June 20.—Killed. Mammary glands invisible, uterus enlarged and congested, with proliferation of mucous membrane.

*Experiment* 4. May 16, 1904. Virgin rabbit received 13 injections of the saline extract of 26 ovaries between May 16 and June 1.

June 1.—Killed. The uterus slightly congested. No effect on mammary glands.

### II.—*Uterus and Uterine Mucous Membrane.*

*Experiment* 5. May 30, 1904.—Virgin rabbit, about six months old, injected with chloroform water extract of the mucous membrane of four pregnant uteri. During 14 days it received four injections.

It was killed on June 20. No changes, either of mammary glands or of uterus.

*Experiment* 6. October 12, 1904.—Two virgin rabbits received during a period of 19 days seven injections of the filtered saline extract of the mucous membrane of seven pregnant uteri. One rabbit (A) received the boiled extract, the other rabbit (B) received the unboiled extract. Rabbit A was killed on October 31. The uterus and ovaries were congested (the animal was in heat), but there was no growth of the mammary glands.

Rabbit B, killed on November 2. The mammary glands showed no change, but the uterus in this one was also congested.

Two other experiments were made on the injection of uterine mucous membrane, but with no result.

### III.—*Injection of Placenta.*

*Experiment* 7. May 15, 1904.—A virgin rabbit received the unboiled extracts (press juice) of the mucous membrane of 14 pregnant uteri and 123 placentæ. These were given in 14 injections between May 15 and June 1. It was killed on June 2. The mammary glands were unaltered. The uterus was congested.

*Experiment* 8. October 12, 1904.—Two virgin rabbits, A and B.

A received on eight occasions during 19 days the boiled extract of 55 placentæ. It was killed on October 31. No results either on uterus or mammary glands.

B received the unboiled extract of 55 placentæ. It was killed on November 2. Mammary glands unchanged ; uterus slightly congested.

### IV.—*Injection of Fœtus with or without Placenta and Uterine Mucous Membrane.*

The first three experiments on this point were also fruitless so far as regards any change in the mammary glands. In these cases the rabbits received from five to seven injections of extract of fœtus. The following experiments, therefore, were conducted for a longer period of time, and a much larger number of injections were given, and in these positive results were obtained.

*Experiment* 9.—February 8, 1905.—A rabbit, eight months old, which had been in the laboratory since it was a fortnight old—virgin—received, *subcutaneously*, extracts made by rubbing up fœtuses and their placentæ with sand, extracting with normal saline, centrifuging, and filtering through a Berkefeld. The injections were made as follows :—

February 8.—Half the extract of 7 fœtuses at the 20th day.
,, 9.— ,, ,, 7 ,, 20th ,,
,, 10.—4 fœtuses at the 22nd day.
,, 11.—5 ,, 14th ,,
,, 13.—4 ,, 14th ,,
,, 14.—3 ,, 22nd ,,
,, 15.—4 ,, 20th ,,
,, 16.—Half the extract of 8 fœtuses at the 20th day.
,, 17.— ,, ,, 8 ,, 20th ,,
,, 18.—6 fœtuses at the 19th day.
,, 20.—5 ,, 14th ,,
,, 21.—Half extract of viscera of 6 fœtuses at the 25th day.
,, 22.— ,, ,, 6 ,, 25th ,,
,, 23.—Viscera of 7 fœtuses at the 20th day.
,, 24.— ,, 4 ,, 24th ,,

The rabbit was killed on February 25. There was marked hypertrophy of the mammary glands, which were surrounded with a certain amount of fluid remaining from the subcutaneous injections. The ovaries were large, the uterus congested, and the vulva showed signs of heat. On microscopic examination there was found to be marked hypertrophy of ducts, which were branching and lined with many layers of cells. The mitotic figures were numerous. Specimens of the mammary glands from this rabbit were shown at the meeting of the Physiological Society at University College in March, 1905.

*Experiment* 10. February 27, 1905.—Intraperitoneal injection of press-juice of the viscera of fœtuses, together with placentæ and uterine mucous membrane.

A virgin rabbit received the following injections :—

February 27.—8 fœtuses, etc., at 24th day.
,, 28.—Half of 9 fœtuses, etc., at 24th day.
March 1.— ,, 9 ,, 24th ,,
,, 2.—8 fœtuses, etc., at 25th day.
,, 3.—8 ,, ,,
,, 4.—2 ,, ,,
,, 6.—6 ,, ,,
,, 7.—4 ,, ,,

Interval of 10 days, owing to failure of supply of pregnant animals.

| | | | | | |
|---|---|---|---|---|---|
| March | 17.— | 7 | fœtuses, etc., at | 16th | day. |
| „ | 21.— | 5 | „ | 26th | „ |
| „ | 23.— | 8 | „ | 22nd | „ |
| „ | 24.— | 14 | „ | 14th | „ |
| „ | 25.— | 12 | „ | 20th | „ |
| „ | 27.— | 5 | „ | 20th | „ |
| „ | 28.— | 8 | „ | 22nd | „ |
| „ | 29.— | 5 | „ | 21st | „ |
| „ | 30.— | 7 | „ | 17th | „ |
| „ | 31.— | 4 | „ | 20th | „ |
| April | 1.— | 9 | „ | 13th | „ |
| „ | 3.— | 9 | „ | 18th | „ |
| „ | 5.— | 5 | „ | 15th | „ |
| „ | 6.— | 11 | „ | 16th | „ |
| „ | 7.— | 6 | „ | 18th | „ |

This rabbit, therefore, received the fluid extracts of the viscera of 160 fœtuses. It was killed on April 8. On reflecting the skin of the abdomen, the mammary glands were seen to be markedly hypertrophied. The margins were almost contiguous, and were somewhat raised and pink, presenting, therefore, much the appearance which is seen in a pregnant rabbit of the eighth or ninth day. The general aspect of the stained gland is shown in fig. 4. On microscopic section not only was there marked duct proliferation with mitotic figures, but at the thickened border the formation of alveoli was just commencing. The whole of these injections had been made intra-peritoneally, or, on one or two occasions, under the skin of the legs, so that there was no infiltration of the connective tissue surrounding the mammary glands. This is the best result which we obtained.

*Experiment* 11. May 2 to June 1, 1905.—In this experiment we sought to determine whether the growth-producing substance is contained chiefly in the viscera or in the body, *i.e.*, muscles, skin, and bones, of the fœtus, and, moreover, whether it could be extracted from these tissues by boiling. Unfortunately, however, out of the four rabbits which we chose for this experiment, only two were definitely virgin, so that the results in the other two cases were equivocal.

Rabbit 1, not a virgin, received the pressed juice of the viscera.

Rabbit 2, a virgin, received the filtered boiled extract of viscera.

Rabbit 3, also a virgin, received the pressed juice of the bodies of the fœtuses unboiled.

Rabbit 4, which was evidently multiparous, received the boiled extract of the bodies of fœtuses. From this rabbit, at the commencement of the experiment, a small portion of mammary gland was taken as a control.

All four rabbits received portions of 182 fœtuses of all ages between May 2 and May 31.

From Rabbits 1 and 4 milky fluid could be expressed from the nipples after the ninth injection.

In Rabbit 2 a watery fluid could be expressed from the nipples after the seventeenth injection.

Rabbit 3 showed traces of watery secretion after the twelfth injection.

All four were killed on June 1. Results were as follows :—

*Rabbit* 1.—Multiparous. Mammary glands well developed and showing many alveoli on microscopic section. The ducts were full of milk ; they were, however, lined with only a single layer of epithelium, and it was impossible to say that any hypertrophy had taken place.

Rabbit 2.—Virgin. The mammary glands did not present much enlargement as judged from inspection. On microscopic examination, however, many branching ducts were observed lined with two layers of cells, presenting the same appearance, but in a smaller degree, as those in the glands of the rabbit in Experiment 10.

Rabbit 3.—Virgin. Glands large, hypertrophied, containing a fair amount of watery fluid. Alveoli present and ducts showing proliferation.

Rabbit 4.—Not virgin. Mammary glands fully marked, and distended with milky fluid, but impossible to determine whether or not hypertrophied.

In order to be certain of the induction of growth in the mammary gland by the injection of extracts of fœtus, three more experiments were made. In the first of these, in which the rabbit received 16 injections of the pressed juice of the viscera of 138 fœtuses, the results were absolutely negative. In this experiment, however, we had been obtaining very small amounts of pressed juice from the tissues, and we thought that the absence of result might possibly be due either to retention of the active substance by the Kieselguhr or to insufficient destruction of the cells in the process of grinding. It is possible, too, that immaturity of the rabbit may have been in some measure responsible for the negative result.

In the next two experiments, therefore, we abandoned the Büchner method and, after grinding with sand and with normal salt solution, centrifuged and filtered the supernatant liquid through a Berkefeld candle before injection. Both these experiments gave positive results.

*Experiment* 12.—October 4 to 21. Virgin rabbit, full-grown. Received daily, intraperitoneally, the saline extract of the viscera of a number of fœtuses about the fifteenth to twentieth day of pregnancy. Killed on the 21st. It showed distinct growth of the mammary glands with duct proliferation (*vide* fig. 5).

*Experiment* 13.—October 4 to 21. Virgin rabbit. Received the saline extract, intraperitoneally, of the bodies and placentæ of the same fœtuses used in Experiment 12. Fifteen injections were given in the 17 days. Killed on the 21st. It showed marked growth of mammary glands with plentiful mitotic figures. The appearance of this gland in the stained specimen is shown in fig. 6.

## *Discussion of Results.*

From the results just described, it will be seen that in six cases we succeeded in producing in virgin rabbits a growth of mammary glands similar to that occurring during the early stages of pregnancy, and consisting in the proliferation of the epithelium lining the ducts, with the multiplication of these ducts by branching into the surrounding tissues. In one of these (Experiment 10) where our injections were carried out during five weeks and the experiment lasted nearly seven weeks, there was an actual formation towards the periphery of the gland of secreting acini. In some of these cases, however, namely those in which the injections had been given under the skin of the back (*e.g.*, Experiment 9), the mammary glands were bathed for considerable periods of time in the injection, and it seemed to us possible that this might be a determining factor in producing growth.

We therefore carried out a control experiment on a virgin rabbit, in which normal rabbit's serum was injected, for the most part subcutaneously, for a period of three weeks. The serum, which was derived from non-pregnant animals, but contained much more nutrient material, *e.g.*, proteid, than the fluids used in our previous injections, ran down in the subcutaneous tissue, so that during the whole duration of the experiment the abdominal wall was thickened and œdematous through the presence of the serum. On killing the animal at the end of three weeks the glands were little, if any, larger than those usually obtained from a virgin animal (fig. 7). On section, however, mitoses were present in the epithelium of the ducts, and there was apparently a certain amount of proliferation of the ducts.

We must conclude, therefore, that superabundant supply of nutrient material in the fluid surrounding the acini may lead to proliferation resembling in kind that which was produced by our injections. This result had not been produced in an earlier control experiment, in which we injected the saline extract of liver, and in view of the small results produced by the injection of the serum as compared with those produced by the injection of the extracts of fœtus much poorer in proteids, we are inclined to believe that it is impossible to explain our results in the other experiments as due to the infiltration of the tissues round the glands. This explanation, at any rate, could not hold for the growth in Experiment 10, in which there had been at no time any injection into the subcutaneous tissue of the back. It is interesting, from the general pathological point of view, to note that typical epithelial proliferation in the ducts can be produced by an abnormally large supply of proteid in their surrounding lymph, and the subject is worthy of further investigation.

A striking fact in all our experiments with a positive result is the smallness of the growth produced as compared with the quantity of material used for injection. In all the positive cases the material for injection was derived from fœtuses; in Experiments 11 (2) and 12 from the viscera only; in Experiment 11 (3) from the bodies only; in Experiments 9 and 13 from the fœtuses together with placentæ; and in Experiment 10 from the fœtuses, placenta, and mucous membrane of uterus together. On the other hand, injection of extracts made from ovaries, uterus, or placenta alone had no effect on the growth of the gland. We are therefore justified in concluding that under normal circumstances the hormone which is responsible for the growth of the mammary gland during pregnancy is produced mainly in the growing embryo. This hormone, however, must be produced in minimal quantities. It is apparently not stored up in any of the tissues of the fœtus or of the placenta, so that, in injecting extracts of fœtus, we are simply injecting the small amount of material which is diffused through the juices on its way to the blood-vessels and into the maternal blood.

It is possible, of course, that the specific mammary hormone is produced from a precursor or mother-substance in some organ or other, and that future research may reveal some method of splitting off the hormone in large quantities, and also of determining whether its production is diffused throughout all the tissues or is confined to one special organ of the body. Injection of extract of duodenal mucous membrane, for example, would give only minimal effects on the pancreas. We should not be justified in concluding from this absence of result that the duodenum was not the seat of origin of the chemical stimulus to the pancreas. Its peculiar relation to the pancreas is only brought into prominence when it is treated with acid, so as to liberate the secretin from its mother substance.

Our experiments, therefore, throw no light on the seat of production of the hormone in the fœtus. Apparently the extent of the growth obtained is a function of the quantity of tissue used in preparing the extracts. The widespread occurrence of the substance in the body of the fœtus points to its being extremely diffusible, as indeed we should expect from analogy with other hormones.

We can only say, therefore, that the hormone is produced by some or all the tissues of the fertilised ovum, whence it is carried off by the blood to the placenta, and so makes its way by diffusion into the maternal blood-vessels. Whether it is identical with the substances which are responsible for the production of the other changes associated with pregnancy, or whether there are distinct substances acting on each organ which is modified during this condition, our experiments do not show. But we have evidence that in the fœtus itself the hormone or hormones of pregnancy have the same result as in the maternal organism. Thus there is increased growth of the mammary glands in the fœtus during the last month of pregnancy, and also in the female an increase in the uterine mucous membrane, as has been shown by Halban. After birth the mammary glands may begin to secrete just as after pregnancy, and there are changes in the uterine mucous membrane similar to those associated with menstruation.

Are we to regard, then, the fœtus as the only source of this hormone? The facts mentioned at the beginning of this paper show that such a conclusion is impossible. The growth of the mammary glands which occurs at puberty can only be ascribed to ovarian influence, and is absent if the ovaries have been previously removed, and Halban ascribes to this ovarian substance both the growth of the mucous membrane during each pro-œstrus and the swelling of the glands at each œstral period, which may in rare cases be attended or followed by the actual formation of milk. Halban explains in the same way those cases recorded by Heape and Kehrer, in which bitches, which had not been impregnated at the normal time, have, after two months, not only made a bed for their young, but have had swelling of the mammary glands, with, in some cases, actual secretion of milk. He would regard this condition as being a continuance of the state of pro-œstrus leading to continued growth of mucous membrane and also of the mammary gland. When the impregnation was no longer possible, with the discharge of the ovum, the secretion of this substance ceased, and the absence of the inhibitory stimulus caused break-down of the uterine mucous membrane as well as dissimilative activity of the mammary gland.

During sexual life, therefore, the ovaries are continually producing a substance which exerts an influence on both glands and uterus. With the occurrence of conception there is at once a great growth of what we may call germinal material. With the growth of the fertilised ovum the amount of hormone produced in the ovum must also increase in proportion. In the early stages of pregnancy the chief source of this hormone may perhaps be located in the chorionic villi, but with the growth of the body of the fœtus this latter must take a preponderating share in the preparation of the hormone. We have no reason to suppose that the fœtal elements of the placenta entirely lose this function of the germinal cells, but the negative results of injection of placentæ in our experiments show that it is impossible to ascribe to the placenta, as is done by Halban, a preponderating part in the preparation of this hormone.

If the hormone is produced in the body of the fœtus, it might be objected that the formation should go on after birth, and therefore lead in the new-born animal to a continuance of the growth both of the mammary gland and of uterus. The profound changes in the environment of the new animal which occur at birth must, however, induce equally profound changes in its metabolism, and there is no difficulty in imagining that with the assumption of extra-uterine life the formation of this substance in the fœtus comes to an end.*

The occurrence of growth in the mammary gland of a virgin rabbit and of secretion in the mammary gland of a multiparous rabbit from the injection of boiled extracts of fœtus, seems to indicate that the specific hormone, like adrenalin or secretin, is not destroyed by boiling. Further evidence, however, is required on this point, as also on the question whether the substance is specific to the animal, or whether the injection of extracts of the fœtus of one animal would produce a growth of the mammary glands in another species. One experiment, in which we fed a kitten for three weeks on the fœtuses of rabbits, was negative in its results. This might, however, have been due to the failure of the intestine to absorb the hormone without destruction, or to the failure of the immature glands to react to the minute stimulus which they received. So far as we know, secretin is not absorbed into the circulation when introduced into the stomach or intestine, and colossal doses of adrenalin have to be given by the mouth in order to produce any systemic effects.

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* In the ornithorhynchus pregnancy is associated with the growth of mammary glands, although the embryo in this animal is contained in an egg, and does not enter into any anatomical connection with the uterine wall. Halban points out, however, that the shell of the egg is porous, and that during its stay in the uterine cavity it increases in size, and the contained embryo grows, in consequence of the absorption of nutrient material from the fluid contained in the uterine cavity. If the embryo is able to absorb nutrient material from the uterine contents, it is equally able to give up to these contents diffusible substances, which may be taken up by the mucous membrane and carried by the circulation to the mammary glands. The condition in the ornithorhynchus cannot therefore be regarded as a disproof of the chemical theory which we have adopted throughout this investigation.

The effect of the injections of fœtal extracts on multiparous rabbits deserves some further mention. The multiparous rabbit differs from a virgin rabbit in possessing ready-formed alveoli, *i.e.*, secretory structures. On the theory which we have adopted, the circulation of the mammary hormone should diminish any secretion in these alveoli and should cause growth. In all our experiments at least 24 hours elapsed between each two injections. It is probable that the hormone was rapidly absorbed from the injection, and was therefore present in the blood of the animal only for a certain fraction, say a few hours, out of the 24. While it was circulating it should cause building up of the secreting cells. Directly, however, it ceased to circulate, the cells would enter into dissimilative activity resulting in secretion. By our injections, therefore, we are not able to imitate the continuous stimulus of pregnancy. We are rather producing each day a pregnancy of a few hours followed by a parturition. These factors should therefore result in the production of milk in any animals possessing the structures (*i.e.*, the alveoli), which are capable of secreting milk, and would therefore account for the secretion of milk observed by us in all the cases where multiparous rabbits were the object of our experiment.

## CONCLUSIONS.

So far as our experiments go, they show that the growth of the mammary glands during pregnancy is due to the action of a specific chemical stimulus produced in the fertilised ovum. The amount of this substance increases with the growth of the fœtus, and is therefore largest during the latter half of pregnancy. Lactation is due to the removal of this substance, which must therefore be regarded as exerting an inhibitory influence on the gland cells, hindering their secretory activity and furthering their growth. It is probable that the specific substance is diffusible, and will withstand the boiling temperature.

We cannot, however, claim that these conclusions of ours are firmly established. A final decision can only be given by a research carried on under more favourable conditions. One requires, in fact, a farm, where we could have at our disposal 500 rabbits, and could arrange for a plentiful supply each day of rabbits about the middle of pregnancy. Under these conditions it might be possible to determine both the seat and nature of the effective stimulus, as well as to test the influence of various reagents in splitting off the hormone from some possible precursor. Many of our experiments, carried out in a London laboratory, were brought to a premature conclusion by failure of material. If, however, the conception of the action of the mammary hormone, which was put forward by Hildebrandt and adopted by us, is correct, namely, that it is a substance which produces growth by inhibiting the normal activity of the gland cell, it should be possible to decide many questions affecting it by working on an animal, such as the goat, in lactation. Injection of the hormone should diminish or stop the secretion of milk while it was circulating in the blood, but should, as a secondary effect, produce an increased secretion as a reaction from the immediate assimilatory effect. The injection might, indeed, have to be prolonged for one or two days, since we know that in Man the onset of a renewed pregnancy during lactation stops the flow of milk only after some time (three or four weeks). At any rate, such experiments could be more rapidly carried out than those which have been the subject of this communication.

## DESCRIPTION OF PLATE.

The drawings were made as follows:—The mammary glands were dissected out, pinned on corked rings, hardened in corrosive sublimate and formol, washed, and stained in very dilute hæmatoxylin. They were then dehydrated, cleared, and mounted as lantern slides in canada balsam between glass plates. (These specimens were shown by projection at the meeting of the Royal Society, on March 1, 1906.) An image of the specimens was thrown (without magnification) on to a piece of millboard, and the darkly stained glands were traced out in indian ink. The figures, therefore, reproduce the glands in natural size.

Fig. 1.—Gland from virgin rabbit.

„ 2.—Mammary gland from primiparous rabbit, five days after impregnation.

„ 3.—Mammary gland from primiparous rabbit, nine days after impregnation.

„ 4.—Mammary gland from virgin rabbit which had received injections of extracts of fœtuses, uterus, and placentæ during five weeks (Exp. 10).

„ 5.—Mammary gland of virgin rabbit, showing growth produced by injection of extracts of fœtal viscera during a period of 17 days.

„ 6.—Mammary gland of virgin rabbit, showing growth produced by injection of extracts of fœtal bodies and placentæ over 17 days.

„ 7.—Mammary gland of virgin rabbit, showing slight growth induced by daily subcutaneous injection of rabbit's serum (from non-pregnant rabbits) during a period of three weeks.

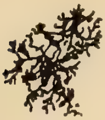


Fig. 1.

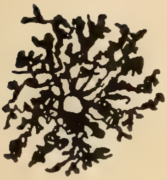


Fig. 2.

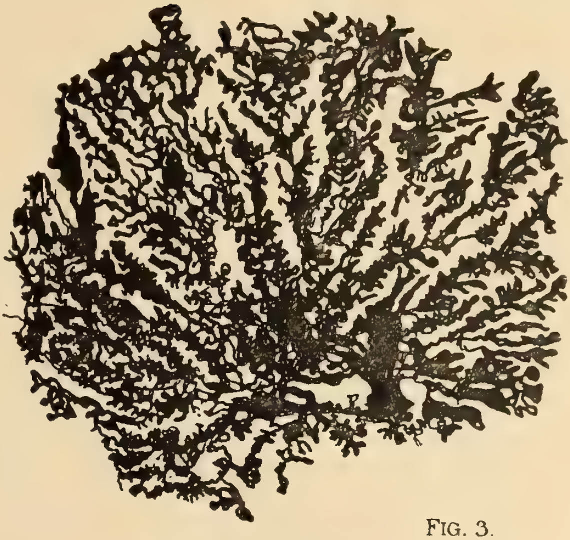
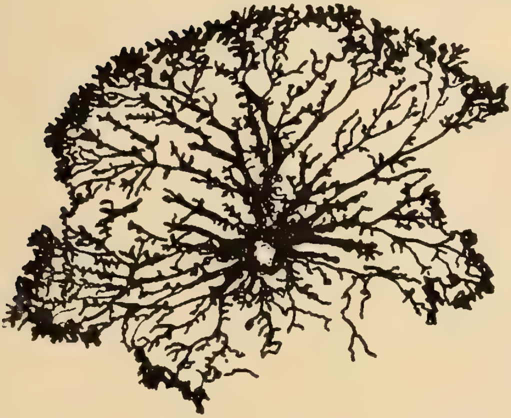


Fig. 3.



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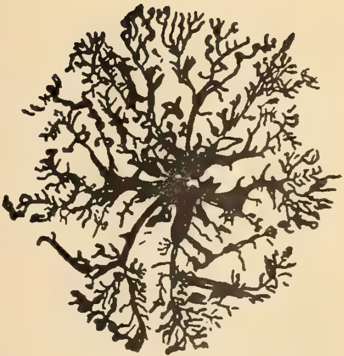
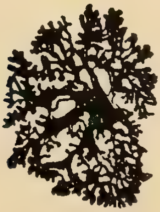


Fig. 5.



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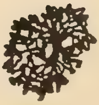

Fig. 7.

# *The Internal Anatomy of Stomoxys.*

By F. Tulloch, Lieut. R.A.M. Corps.

(Communicated by Professor E. Ray Lankester, F.R.S. Received February 2,—Read March 1, 1906.)

The dissections of the local variety of Stomoxys, which form the subject of this Note, were made at the suggestion of Professor Minchin, during his direction of the Royal Society's Commission on Sleeping Sickness in Entebbe, Uganda. The main object was to furnish some comparison between the internal anatomy of Stomoxys and that of Glossina, and the following notes are based on Professor Minchin's description of *Glossina palpalis.*

Complete digestion of the human trypanosome seems to occur in 48 hours, in the alimentary canal of Stomoxys; but Lieutenant Gray, R.A.M.C., has found a limited percentage of these Stomoxys to be infected with a Herpetomonas.

I am much indebted to Professor Minchin for advice and assistance at every turn, without which these notes could not have been completed.

## *Digestive System.*

The œsophagus emerges from the chitinous pharynx (which, with the mouth parts, has been described by Hansen) as a flattened tube, which gradually narrows and becomes cylindrical, running at first upwards and then backwards to reach the brain. The connectives of the brain are more vertical than in Glossina. On emerging from their constriction the œsophagus dilates gradually, and runs down to enter the ventral aspect of the proventriculus, which lies in the anterior third of the thorax.

The proventriculus (fig. 1, P.) is a mushroom-shaped viscus with a thickened border, and lies with its convexity pointing upwards and slightly forwards. Except for the inversion of its lateral edges, which gives to the proventriculus of Glossina a characteristic outline, the corresponding structure in Stomoxys is very similar in every way. The œsophagus enters the proventriculus a little in front of the centre of its concave ventral surface, and the duct of the sucking stomach running up from below appears to enter with it, though in reality it enters separately at a point immediately behind. As in Glossina, the œsophagus and the duct of the sucking stomach are in the same line.

The thoracic intestine (fig. 1, T.I.) arises from the convex dorsal surface of the proventriculus at a point posterior to the entrance of the œsophagus on the ventral surface. From its origin the intestine runs down into the abdomen of the fly as a narrow tube of uniform diameter, until it reaches nearly to the lower border of the sucking stomach. At this point it dilates to several times its former diameter, its wall at the same time becoming thinner.

The abdominal intestine is proportionately shorter, less coiled, and more distensible than in Glossina; it is about three times as long as the fly itself. The dilated portion of intestine has three simple coils which lie superposed in the middle part of the abdomen, and then gradually narrows, continuing as a uniformly narrow tube down to the rectum. The narrow lower intestine has variable bends in its course, but is not coiled.

The rectum (figs. 1 and 2, R.) is a dilated cone-shaped portion of intestine, the apex of the cone being towards the anus. Its walls are transparent, and through them are readily seen four long trumpet-shaped papillæ, so-called rectal glands, the narrow ends of which are inserted towards the anus (fig. 2, R.P.). A single trachea enters the base of each "gland." Below the apex of the dilated cone the rectum is continued to the anus as a short narrow tube. In the female this terminal portion of intestine runs within the ovipositor, the anus being situated between the last segment of the ovipositor and the terminal plate. In the male the ejaculatory duct passes over it dorsally from left to right, and runs anteriorly to enter the penis.

The appendages of the alimentary canal are the Malpighian tubes, the sucking stomach, and the salivary glands.

The Malpighian tubes (fig. 2, M.T., M.T.) arise from the narrow lower intestine. The proctodæum, between their origin and the anus, comprises in length about one-fifth of the abdominal intestine. At their point of origin (figs. 1 and 2, O.) the intestine has a shallow linear constriction. Two tubules arise on each side from a short common tube, and all four tubules are approximately of the same length. The two tubules arising from one side have thickened terminations (fig. 2, T.T.), some four times greater than a salivary gland, and these thickened endings lie in the pericardial sinus. The tubules of the other side are of the same thickness throughout, and their ends lie amid the fat-body of the lower abdomen. Microscopically the tubules are of the usual type.

The sucking stomach (fig. 1, S.S.) is a thin-walled sac, made up of one layer of flattened cells with occasional strands of unstriped muscle. It ends at the waist in a very fine duct (D.S.S.) which runs up ventrally to the thoracic intestine and enters the proventriculus (P.) immediately behind the opening of the œsophagus. The alimentary canal and the ducts in the thorax lie in contact with each other in a narrow space between the lateral masses of thoracic muscles.

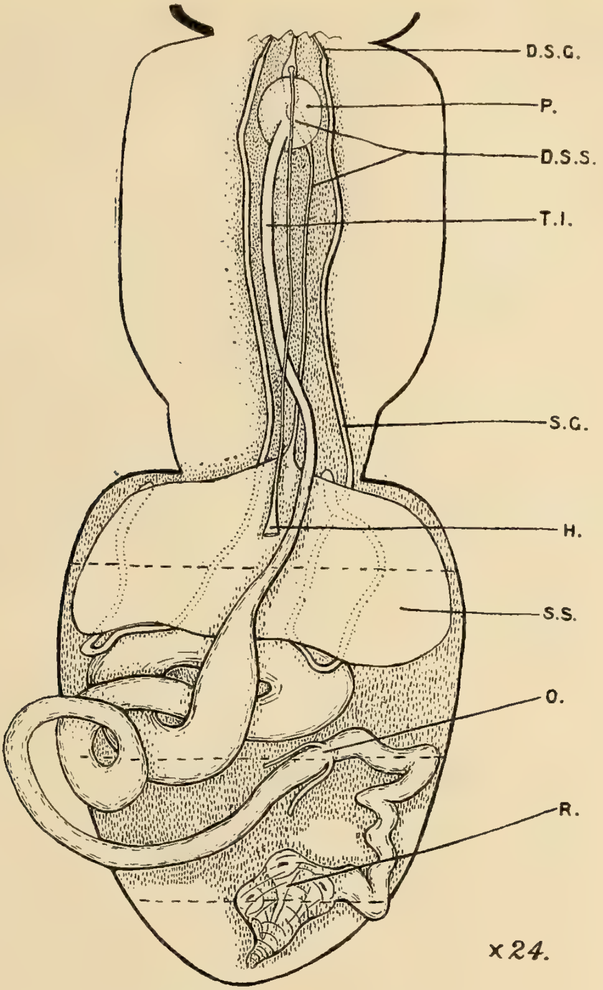

Fig. 1.—Alimentary Canal of Stomoxys. Dorsal view. The thoracic muscles were removed, and the structures in the thorax separated and spread out, though their relative positions are otherwise maintained. In the abdomen the position of the coils of intestine has been very little disturbed, but the Malpighian tubes have been removed by severing their common ducts on each side near O. P., proventriculus; T.I., thoracic intestine; R., rectum; D.S.G., duct of salivary gland; S.G., salivary gland; S.S., sucking stomach; D.S.S., its duct; O., point of origin of Malpighian tubes; H., the dorsal blood vessel cut short at the heart.

The salivary glands (figs. 1 and 2, S.G.) are partly thoracic and partly abdominal. They are comparatively shorter and thicker than in Glossina. In the abdomen they are ventral to the sucking stomach, and from a dorsal view only a knuckle of gland is exposed at the lower border of this viscus.

The slightly bulbous ends of the glands lie under the upper border of the sucking stomach, and are found by following up the outer limb of the exposed angle of gland. Except for this angular bend the glands are straight in their whole course, and even when pulled out they are not long enough to reach the hinder end of the fly.

The salivary glands run up through the waist of the fly on either side of the duct of the sucking stomach and ventral to the intestine, and continue with the same thickness to the front of the thorax. At this point, in the neck of the fly, the glandular portion ceases abruptly, to be continued as a very fine narrow duct (fig. 1, D.S.G.). At first this duct is made up of small flattened cells, but it almost immediately acquires the structure of a small trachea, becoming chitinised and having similar annular thickenings. At the base of the brain the two ducts join and continue as a single duct on the ventral surface of the chitinous pharynx, inside the transparent membrane which wraps it round. The dilatation in the common duct which Hansen has described and which he regards as a storage chamber for the secretion, occurs about half-way in the length of the common duct. The point of entrance of the duct into the proboscis has been described by Hansen.

### *Nervous System.*

This consists of the brain and the thoracic ganglion, with the nerves arising from them.

The nervous system was not dissected in great detail, but the following nerves were traced as described. The thick nerve to the ocelli arises from the upper part of the back of the brain. The stout nerves to the antennæ arise from the front of each cerebral ganglion. On either side of the front of the brain below the nerves to the antennæ arises the slender pharyngeal nerve trunk, which shortly divides into three. The outermost of these three branches divides into two, one filament supplying the depressor muscle of the pharynx which arises from the postero-superior process, and the other running down inside the pharynx in close relation to its chitinous wall. The middle division of the pharyngeal nerve joins its fellow of the opposite side on the wall of the œsophagus as the latter enters the pharynx, the common trunk thus formed splitting into four branches to the intrinsic muscles of the pharynx. The innermost branch of each pharyngeal nerve joins a slender nerve arising in the middle line. The nerve thus formed supplies the pharyngeal muscles, but was not traced in detail.

The brain is connected with the thoracic ganglion by the connectives, between which passes the œsophagus and which join after this to form a long connecting band as in Glossina. The thoracic ganglion is somewhat pear-shaped, and is supported by the internal chitinous skeleton of the thorax, from the surfaces of which arise the wing and leg muscles. Six pairs of nerves arise from the thoracic ganglion and supply the thoracic muscles.

The abdominal nerve trunk continues from the posterior part of the ganglion running down in contact with the abdominal wall. It gives off three fine branches which supply the abdominal muscles, and ends in the third segment of the abdomen by dividing into three. Each of these branches again divides to supply the generative organs, the outer two running to the ovaries or testes and the middle one to the muscles of the ovipositor or penis.

### *Circulatory System.*

This consists of the heart and its continuation, the thoracic aorta. The heart is a tubular organ of the same type as in Glossina with chambers, ostia, and alary muscles. The wall, too, is composed of similar giant cells. Though several stained preparations were made it was impossible, owing to the fat-body which obscured all detail, to count the chambers and cells in the heart wall. They seemed, from a comparison of all the preparations, to be reduced in proportion to the smaller number (four) of abdominal segments possessed by Stomoxys.

The dorsal aorta consists of paired cells, as in Glossina, and runs up on the dorsal surface of the intestine to end on the œsophagus in a similar mass of cells. On the surface of the proventriculus, to which it is bound down, it becomes expanded and flattened, narrowing again to its termination.

### *Male Generative Organs.*

These are comparatively simple. The testes (fig. 2, T.) are a pair of smooth, oval, orange-brown bodies with a shallow equatorial constriction. Their colour is due to a pigmented coat as in Glossina, but there is apparently not the same tubular structure.

From the lower end of each testis arises a very fine duct (D.), short and straight, which runs down to join the duct of the opposite side as the upper limbs of a Y. From this junction an exceedingly short length of common duct (C.D.) runs into the bulbous upper end of a tubular organ, which would seem to function as a vesicula seminalis.

This vesicula seminalis (V.S.) is a flexible tube, often lying with two U-shaped bends in its course. At its upper part it is bulbous, gradually narrowing below this to end as an ejaculatory duct, which crosses the rectum dorsally from left to right, to enter the penis in front of it; it does not thus encircle the rectum as in Glossina. The hypopygium and penis are of the same type as in Glossina.

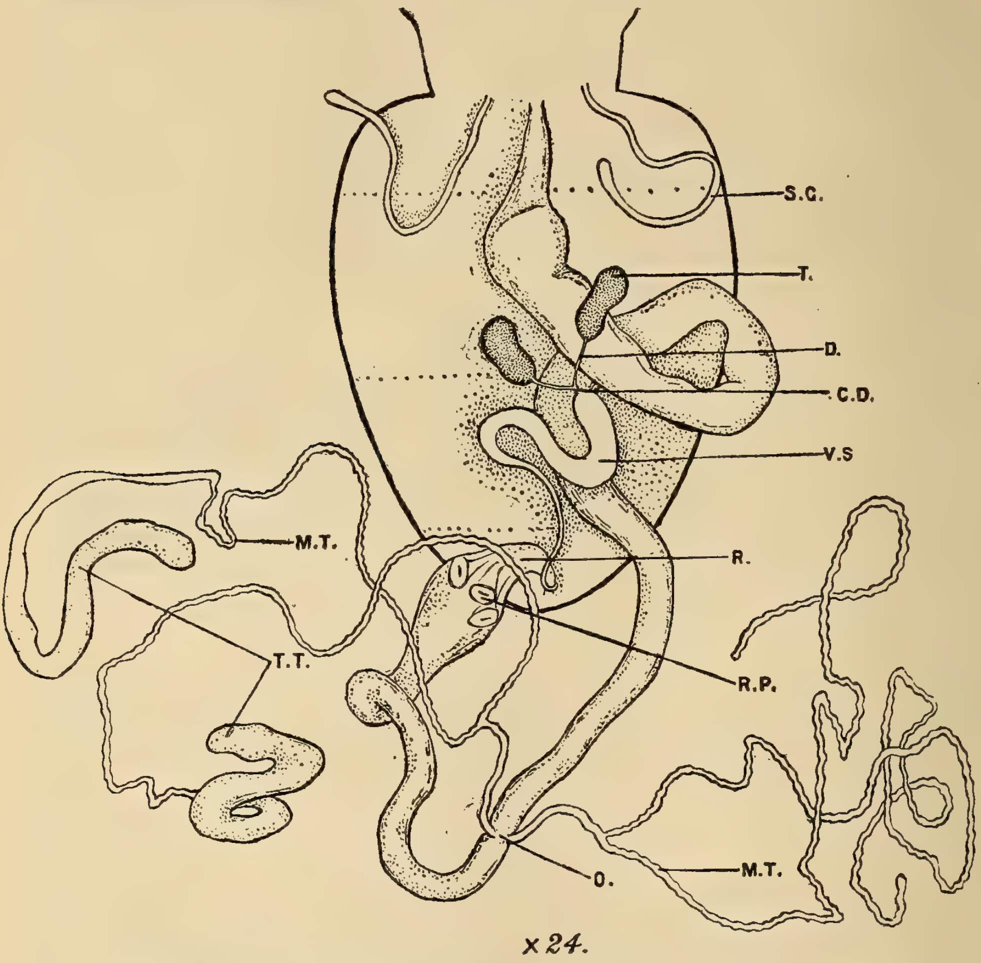

Fig. 2.—Male Generative Organs of Stomoxys. Alimentary canal dissected out to show Malpighian tubes. Dorsal view. S.G., salivary gland; O., origin of Malpighian tubes; M.T., Malpighian tubules; T.T., thickened terminations of the tubules of one side; R., rectum; R.P., rectal papillæ, three of the four are seen through the transparent rectal wall; T., testis; D., duct of testis; C.D., common duct; V.S., vesicula seminalis.

### *Female Generative Organs.*

The sex of a Stomoxys can be easily ascertained by inspection of the hind end of the abdomen; but, unlike Glossina, the scutellar bristles are of the same length in both sexes. The female generative organs are of the house-fly type. There are two ovaries (fig. 3, O., and fig. 4), each consisting of some 60 ovarioles. The ovary is moored to the body wall by a profusely branching trachea, which arises from the pleural space and ramifies among the ovarioles. In the natural position the ovaries lie with the long axis of the ovarioles pointing upwards towards the dorsal surface. Each ovariole contains never more than four ova in various stages of development.

The ovaries vary in size according to the degree of maturity of the lowest ova. In some flies they occupy more than half of the whole abdominal space. The ovarioles open into a wide tubular duct which joins its fellow from the other ovary like the upper limbs of a Y. As a result of this junction is formed the common oviduct (fig. 3, C.O.), which runs down, forming a long third limb to the Y. Below the attachment of the uterine appendages the oviduct continues as the uterus.

The appendages consist of the uterine glands and the receptacula seminis. The uterine glands (fig. 3, U.G.) are two rather stout tubular organs with slightly bulbous extremities. The bulbous end is firmly joined to the lateral oviduct by a very short double strand of connective tissue. Each gland ends in a short fine duct, and these ducts enter separately the shallow constriction which forms the arbitrary division between the oviduct and uterus.

The receptacula seminis (fig. 3, R.S.) are two small, black, spherical bodies, each with a cellular socket resembling the fitting of an acorn cup. From this runs a very fine duct which enters the division between the oviduct and uterus in the mid-dorsal line. The receptacula are attached to each other, but can be separated by dissection. The distal portions of the two ducts are quite separate, but later each duct enlarges slightly, and from this point on to its insertion is closely attached to its fellow. This portion can, however, be separated by dissection, and it is then seen that the ducts are distinct and enter separately.

The uterus (fig. 3, U.) is a tube of the same diameter as the common oviduct above, and runs down in the middle line into the ovipositor. The ovipositor (fig. 3, O.P., and fig. 4) consists of three cylindrical segments of thin chitin, which usually lie telescoped inside the abdomen. There is also a single external flap of dark chitin which lies folded up on the ventral surface of the fly. When the ovipositor is extruded by squeezing the fly's abdomen, the receptacula and uterus are pulled down with it, and can be seen through the transparent walls.

The upper segment of the ovipositor has three narrow ribs of dark chitin in its long axis, two dorsal and one ventral. The next segment is similar. The last segment has two dorsal plates only. The external flap, which is probably the third rib of the last segment, is, roughly, quadrilateral, and has two divergent prong-like processes arising from its free border.

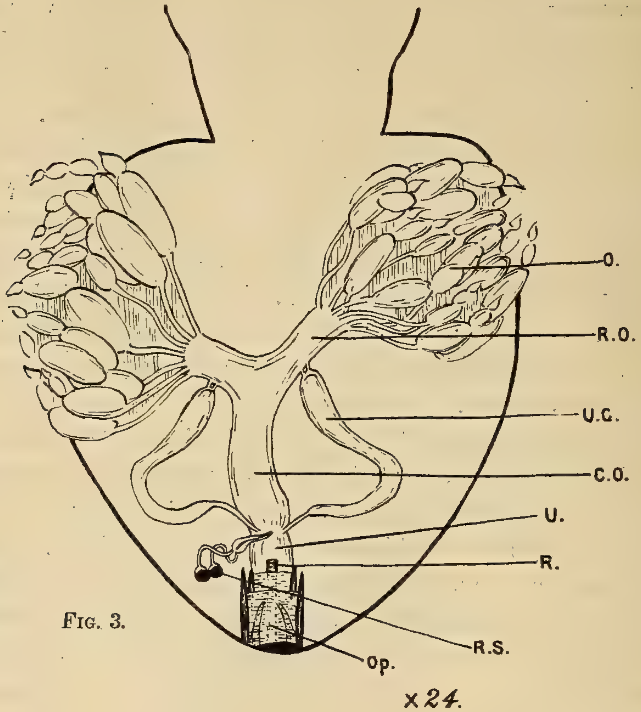

FIG. 3.

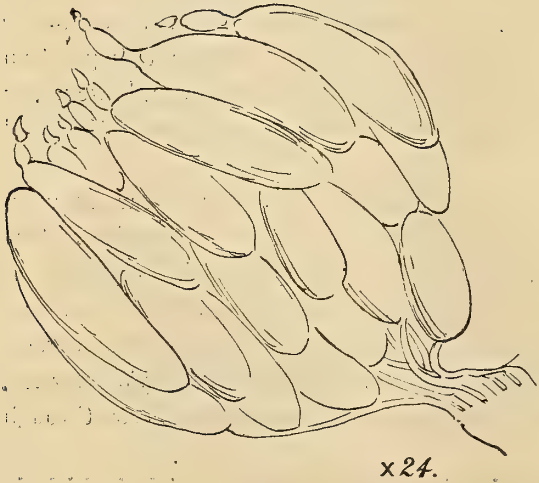

FIG. 4.

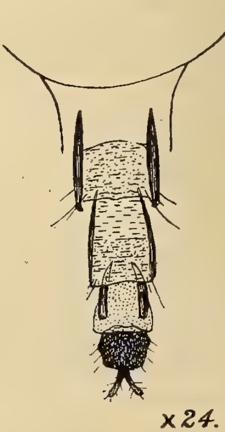

FIG. 5.

FIG. 3.—Female Generative Organs. The natural position of the parts has been considerably altered for the sake of clearness. Dorsal view. O., ovary; R.O., right oviduct; C.O., common oviduct; U., uterus; O.P., ovipositor; U.G., uterine gland; R.S., receptacula seminis; R., terminal position of rectum cut short above.

FIG. 4.—Mature ovary from another specimen.

FIG. 5.—Ovipositor extended. Dorsal view. The ventral ribs of chitin in the two upper segments are not shown.

The points of the chitinous ribs which strengthen the segments of the ovipositor project above the upper border of the segment, and to them are attached the muscles of the ovipositor. The narrowed terminal portion of the rectum enters the ovipositor on the dorsal surface of the uterus and runs down to the anal opening between the external plate and the last segment.

# *The Specificity of the Opsonic Substances in the Blood Serum.*

By William Bulloch, M.D., and G. T. Western, M.A., M.B.

(Communicated by Leonard Hill, F.R.S. Received February 15,—Read March 1, 1906.)

(From the Bacteriological Laboratory, London Hospital, E.)

A relatively high degree of specificity has been demonstrated for most of the antibodies which exist in immune sera, *e.g.*, in the case of agglutinins, lysins, præcipitins, antitoxins. With normal sera the proof of specificity is often difficult on account of the fact that the antibodies are present in the majority of cases only in small quantities.

The following experiments are concerned with the specificity of the opsonic substances of normal and immune sera. As is well known, these opsonic substances, discovered by Wright and Douglas, act on bacteria in such a way that the latter become an easy prey to the phagocytic leucocytes.

If a given serum be tested it will be found to exert an opsonic action on more than one kind of bacterium, and the question we have sought to answer is whether there is one or more than one opsonic substance; in other words, whether the opsonins are specific for the different bacteria on which they exert their opsonic action.

In a previous communication* one of us (B.) has shown that when a microbe, *e.g.*, staphylococcus, is digested with normal serum at 37° C. for 15 minutes, and the cocci are then brought down by the aid of a centrifuge, the supernatant liquid is found to be devoid of opsonic action for staphylococci. Where the contact of the microbe with serum has been sufficiently long, and the centrifugalisation has been complete, the opsonin for the particular microbe is totally removed.

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* 'Roy. Soc. Proc.,' vol. 74.

We have attempted to determine whether the opsonins are specific by experiments of two kinds:—

1. The first method consisted in estimating the opsonic content of a given serum towards two different bacteria. A suspension of one of these bacteria was digested with the serum, and the mixture was thereafter centrifugalised, the resulting supernatant liquid being tested on both kinds of bacteria. To a quantity of the supernatant liquid the second bacterial suspension was added, and after the lapse of a certain time the centrifuge was again applied, and the resulting liquid was again tested.

2. The second method consisted in estimating from day to day the opsonic content of the serum of human beings suffering from lupus. At certain periods tubercle or staphylococcus vaccines were inoculated, and the effect on the two opsonic curves was determined.

1. Experiment on the opsonic action of normal human serum towards *Staphylococcus aureus* and *Bacterium pyocyaneum* respectively.

Normal human serum was mixed with an equal volume of a suspension of *Staphylococcus aureus*, and the mixture was placed in the incubator for 1 hour at 37° C. At the end of this time the mixture was centrifugalised, the supernatant liquid "A" being removed from the deposit of cocci by means of a pipette. The supernatant liquid was in part retained, the remainder being digested for 1 hour at 37° C. with a suspension of *Bacterium pyocyaneum*, the latter being finally brought down as a deposit in the centrifuge, leaving a supernatant liquid "B," which was pipetted off.

Result.

| | | | | | | |
|---|---|---|---|---|---|---|
| 1. | Normal serum | (1 in 2 dilution) | + staphylococci | + leucocytes | = 22·9 | Bacteria per leucocyte. |
| 2. | „ „ | (1 in 2 „ ) | + *B. pyocyaneum* | + „ | = 4·7 | |
| 3. | „ „ | (1 in 4 „ ) | + „ | + „ | = 3·0 | |
| 4. | Fluid "A" | | + staphylococcus | + „ | = 0·5 | |
| 5. | „ "A" | | + *B. pyocyaneum* | + „ | = 4·0 | |
| 6. | „ "B" | | + „ | + „ | = 0·4 | |

The contact of the serum with staphylococcus leaves the opsonic action of the serum for *Bacterium pyocyaneum* practically unchanged, the pyocyanic opsonin being finally removed by contact of the serum with this microbe.

A similar result was obtained when the serum was brought to act on staphylococcus and tubercle bacillus, as can be seen in the following experiment.

1. Normal human serum was mixed with an equal quantity of an emulsion of tubercle bacilli in 0·85 per cent. NaCl solution. The mixture was digested for 30′ at 37° C. and then centrifuged. In this way a deposit and a supernatant liquid "A" was obtained.

No. of Microbes per Leucocyte.

| | Expt. I. | | | Expt. II. |
|---|---|---|---|---|
| | B. | W. | Mean. | |
| 1. Normal serum + saline *a.a.* (3 parts) + T.B. (1 part) + leucocytes (3 parts) …… | 3·03 | 3·0 | 3·015 | 1·61 |
| 2. Normal serum + saline *a.a.* ( „ ) + staphylococcus ( „ ) + „ ( „ ) …… | 12·6 | 12·3 | 12·45 | 7·00 |
| 3. Normal serum 1 + saline 3 ( „ ) + T.B. ( „ ) + „ ( „ ) …… | 1·4 | 1·4 | 1·4 | 1·40 |
| 4. Normal serum 1 + saline 3 ( „ ) + staphylococcus ( „ ) + „ ( „ ) …… | 11·0 | 11·4 | 11·2 | 5·20 |
| 5. Fluid "A" ( „ ) + T.B. ( „ ) + „ ( „ ) …… | 0·4 | 0·5 | 0·45 | 0·13 |
| 6. „ "A" ( „ ) + staphylococcus ( „ ) + „ ( „ ) …… | 9·0 | 10·23 | 9·96 | 5·00 |
| 7. „ "B" ( „ ) + T.B. ( „ ) + „ ( „ ) …… | 2·7 | — | 2·7 | 1·20 |
| 8. „ "B" ( „ ) + staphylococcus ( „ ) + „ ( „ ) …… | 0·43 | 0·26 | 0·34 | 0·80 |
| 9. „ "C" ( „ ) + „ ( „ ) + „ ( „ ) …… | 0·13 | 0·26 | 0·19 | 0·40 |
| 10. „ "D" ( „ ) + T.B. ( „ ) + „ ( „ ) …… | 0·16 | 0·76 | 0·51 | 0·32 |
| 11. „ "A" (3 parts) + saline (1 part) + leucocytes (3 parts) (stained for T.B.) ………… | 0·09 | 0·0 | 0·09 | 0·10 |
| 12. „ "B" ( „ ) + „ ( „ ) + „ ( „ ) ( „ staphylococcus) | 0·0 | 0·0 | 0·0 | 0·00 |
| 13. „ "C" ( „ ) + „ ( „ ) + „ ( „ ) ( „ „ ) | 0·0 | 0·0 | 0·0 | 0·00 |
| 14. „ "D" ( „ ) + „ ( „ ) + „ ( „ ) ( „ T.B.) ………… | 0·05 | 0·0 | 0·05 | 0·00 |
| 15. Saline, 0·85 per cent. (3 parts) + T.B. (1 part) + leucocytes (3 parts) ………… | 0·0 | 0·08 | 0·08 | 0·06 |
| 16. „ 0·85 „ ( „ ) + staphylococcus ( „ ) + „ ( „ ) ………… | 0·13 | — | 0·13 | 0·08 |

2. Normal human serum was mixed with an equal quantity of an emulsion of *Staphylococcus aureus* in 0·85 per cent. NaCl solution. The mixture was digested for 30′ at 37° C. and then centrifuged, a supernatant liquid "B" being obtained.

3. The fluid "A" was mixed with an equal quantity of an emulsion of *Staphylococcus aureus*. The mixture was digested for 30′ at 37° C. and a deposit separated from a fluid "C" by the centrifuge.

4. The fluid "B" was mixed with an equal quantity of an emulsion of tubercle bacilli. The mixture was digested for 30′ at 37° C., and a deposit separated from a fluid "D" by the centrifuge.

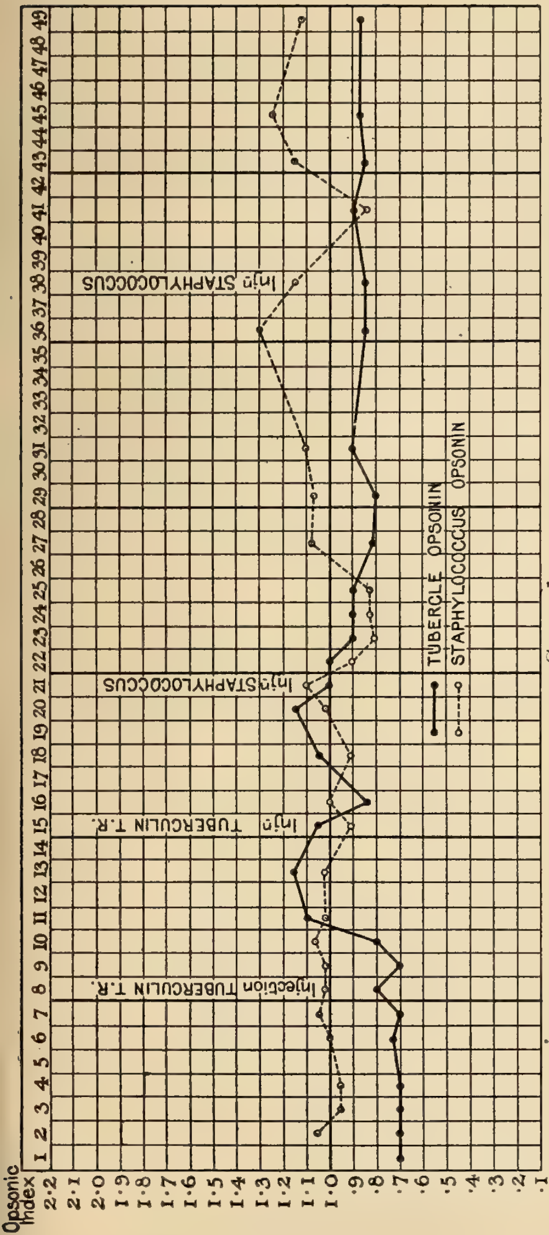
The opsonic content of the serum and of the fluids "A," "B," "C," and "D" was then determined both for *Staphylococcus aureus* and tubercle bacillus in the usual way, the necessary controls being added. In the first experiment the determinations made by each of us separately in a series of different films are given under the designation B. and W., and the mean of these determinations. In the second experiment the result was obtained by one of us (W.) alone.

It will be seen that a considerable degree of specificity exists in so far that staphylococci remove almost the whole of the opsonin for this microbe, while the opsonic substance for tubercle bacilli is in large part left unaltered. In almost all cases we have observed a slight diminution in the quantity of the opsonin left behind. Thus while the contact of a serum with tubercle bacilli lowered the opsonic content for this bacillus from 3·03 to 0·4, it also produced a slight lowering of the staphylococcus opsonin from 11·2 to 9·96. Similarly, contact of a staphylococcus with serum reduced the staphylococcus opsonin from 12·45 to 0·34, and at the same time it lowered the tubercular opsonin from 3·015 to 2·7.

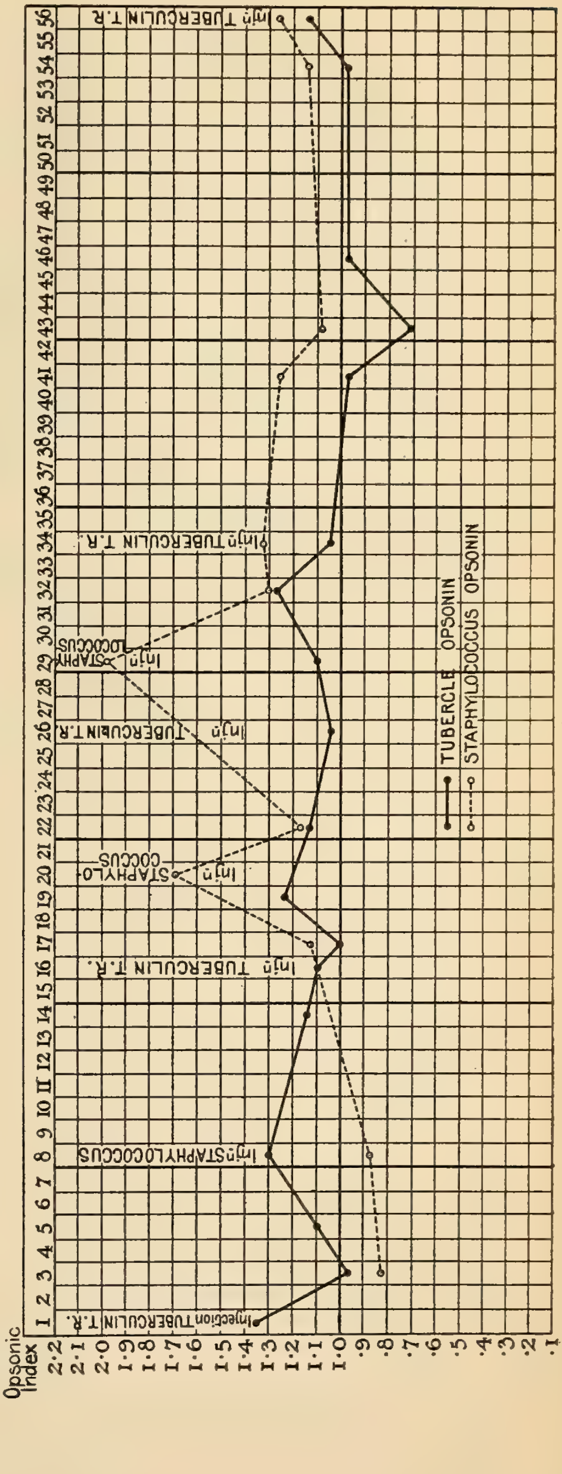
2. Experiment:—

The opsonic content of the serum of a patient suffering from lupus was repeatedly determined on tubercle and staphylococcus suspensions. Two inoculations of tuberculin and two of staphylococcic vaccine were injected, and the influence of the inoculations is set forth in the following opsonic curve, which shows that there is no correspondence in the quantities of tuberculo-opsonins and staphylococcus opsonins when one or other of the corresponding vaccines is inoculated (Case I).

In a second experiment (Case II) opsonic determinations were made in a similar case, with the exception that the patient was not only suffering from lupus, but septic infection of the tuberculous lesions at the same time (Curve 2).



CURVE 1.



CURVE 2.

### *Conclusions.*

1. When staphylococci are brought into contact with normal human serum and are subsequently removed by centrifugalisation, the serum loses its opsonic power for staphylococcus, although the opsonic power of *Bacterium pyocyaneum* is preserved.

2. Contact of normal human serum with tubercle bacilli leaves the opsonic power of that serum for staphylococcus almost intact, while the opsonic power for tubercle bacillus is completely removed.

3. Contact of normal human serum with staphylococcus leaves the opsonic power of that serum for tubercle bacillus almost intact, while the opsonic power for staphylococcus is completely removed.

4. Inoculation of a human being with tuberculin causes quantitative increase in the tuberculo-opsonin, whereas the quantity of staphylococcus opsonin is unaltered.

5. Inoculation of a human being with staphylococcus vaccine causes a quantitative increase in the staphylococcus opsonin, whereas the quantity of tuberculo-opsonin is unaltered.

# *On the Relationship between Hæmolysis and the Phagocytosis of Red Blood Cells.*

By R. D. Keith, M.A., M.D.

(Communicated by Leonard Hill, F.R.S. Received February 15,—Read March 8, 1906.)

The nature of the substance or property in normal as well as in immune serum which induces phagocytosis has been of late a matter of considerable discussion, and the chief point of controversy has been whether phagocytosis is caused by some well-known immune substance, or whether it is brought about by something which until recently had not been completely recognised as a product of immunisation processes, *e.g.*, the "opsonin" of Wright and Douglas.

Whatever the nature of this substance may be, it seems established beyond doubt that it acts on the bodies phagocytosed, the stimulin theory of Metchnikoff and his school having given way to the theory supported especially by Wright and other observers in this country, that the action is on the bodies phagocytosed and not on the phagocytes, notwithstanding the work of Löhlein (1), Leishman (2) and Besredka (3).

Wright and Douglas (4 and 5) in their well-known work on this subject, described this property of the serum as being due to a body which up to that time had not been properly recognised. To this they gave the name "opsonin," and by their ingenious experiments they rendered clear and concrete what had been before but nebulous and ill-defined.

They as well as Bulloch and Atkin (6) and Hektoen and Ruediger (7) described this body as being thermolabile from the fact that it was to a large extent destroyed by heating the serum to 55° C. to 65° C. Dean (8) repeated this work, using a somewhat different technique, and having found that in normal, but especially in immune sera, a certain amount was not destroyed, decided to call it thermostable. As Wright (9) has since pointed out, this is merely a matter of terms; but from his as well as from Dean's experiments it is clear that a very large amount of destruction takes place at these temperatures.

Dean at the same time put forward the view, shared chiefly by workers in the Pasteur Institute in Paris, that the substance or property in the serum described by Wright and Douglas was not new but had been well known before, and Dean laid stress on the work of Savtchenko (10) on the phagocytosis of red blood cells, pointing out that this property had been attributed by Savtchenko to the "fixateur."

As there seems to have crept into this question some doubt as to the exact interpretation to be put on Savtchenko's work, and particularly as to the exact significance of the term "fixateur" as used by him, it is necessary to briefly consider his position, especially as Barratt (11) has put a different interpretation on it from Dean.

Savtchenko assumed that the laws regulating the action of cytotoxins were entirely analogous to those regulating the action of immunising substances on microbes, and considered that experiments on phagocytosis might be permissibly conducted with animal cells and adopted red blood cells as being easy to work with.

This opinion would indicate that Savtchenko considered that the action of hæmolysis was the analogue of that of immunising substances on microbes, since a cytotonic action with reference to red blood cells would mean hæmolysis. This is also indicated further on in his work when he says that as has been pointed out by Bordet, when the red blood cells of an animal A are injected into an animal B, the serum of the latter becomes toxic for the red blood cells of the former, and that he himself has established a complete analogy between the action of the serum on the red blood cells and that of the immune specific serum on the microbe as well in the animal body as "in vitro."

Further he says,* "Dans le sérum spécifique se trouve une substance ou fixateur (d'après la terminologie de Metchnikoff) qui se fixe sur les globules rouges correspondants—ou bien sur les microbes—et par son action prépare ces derniers à leur dissolution par les alexines (cytases) qu'on trouve dans chaque sérum. Le fixateur ne se détruit pas à 55° C. à 60° C. Ehrlich et Morgenroth ont montré que le fixateur a une affinité spécifique pour les globules rouges correspondants, et qu'une fois fixé sur eux, il ne s'en détache pas dans les lavages ultérieurs, ainsi que dans la centrifugation dans l'eau physiologique. Si l'on soumet les globules rouges ainsi traités à l'action du sérum normal contenant des alexines, ils se dissolvent."

With regard to Metchnikoff's definition of the fixateur which Savtchenko accepts, one may state what Metchnikoff (12) himself has given in his latest work on the subject.

On p. 355 he says, "Um in diesen bedeutungsvollen Ergebnissen das sicher Festgestellte und das Hypothetische von einander zu halten, haben wir vorgeschlagen das Alexin oder Komplement unter dem Namen Cytase (d. h. zellenlösendes Enzym), die sensibilisierende Substanz dagegen unter dem Namen Fixator zu bezeichnen." He also states (p. 357) that Savtchenko was the first to show that red blood cells which are laden with *the specific fixateur* are extraordinarily easily phagocytosed.

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* *Loc. cit.*, p. 111.

Savtchenko stated further that he took as the objects of experiment the phagocytes of the guinea-pig and its red blood corpuscles, and the serum of a rabbit immunised against the red blood cells of the guinea-pig, and heated the serum of the rabbit to 55° C. to destroy the alexines, leaving *the specific fixateur* intact. He also stated that he took the washed red cells of a guinea-pig and diluted them with normal saline solution and added a quantity of heated hæmolytic immune serum in a dilution of 1/200. After this mixture had been six hours at 37° C. he centrifugalised and washed the corpuscles thrice with normal saline. "The red blood cells," he adds, "had attached to themselves *the fixateur*; since the addition of normal serum was sufficient to bring about the solution of the hæmoglobin."

Again,* he states, "Il est possible qu'il existe dans le plasma un minimum de fixateur insuffisant pour être decélé par la réaction de dissolution, mais tout à fait suffisant pour provoquer la phagocytose après s'être fixé sur ces derniers."

Savtchenko's position is this: As the result of his experiments he came to the conclusion that in the serum of a rabbit immunised with guinea-pig's blood, there exists a substance which causes phagocytosis of the red blood cells of the guinea-pig, and this substance, which may act either on the phagocytes or on the bodies to be phagocytosed, is the specific fixateur, and possibly, according to the amount present in the serum, this substance causes hæmolysis or phagocytosis.

From what has been given here of Savtchenko's work, it appears to be beyond doubt that he considered that the specific fixateur which induces the phagocytosis of red blood cells is the same as the hæmolytic amboceptor of Ehrlich and not a separate body inducing this action.

Barratt† has shown that even with *unheated* immune serum, phagocytosis of red blood cells may occur without the serum possessing either hæmolytic or agglutinative properties, and concludes from this that the phagocytosis is not induced by the fixateur in the sense of the term as used by Savtchenko, nor by the agglutinin, but by some other body acting on the red blood corpuscles and not on the leucocytes. This body he placed in the class of "opsonins."

Besredka (13) in summing up Barratt's paper says, "Il y a, en effet, dans un sérum hæmolytique plusieurs substances. Est-ce le fixateur (amboceptor), qui détermine la phagocytose? est-ce l'agglutinine? est-ce enfin une troisième substance qui aurait uniquement pour fonction de présider à la phagocytose"? Besredka, it is clear, also assumes that the fixateur is identical with the amboceptor.

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* *Loc. cit.*, p. 118.

† *Loc. cit.*

The main question at issue, then, is whether the amboceptor, and by this I mean that acting in hæmolysis, is identical with the substance inducing the phagocytosis of erythrocytes—the opsonin of Wright and Douglas.

As Savtchenko and Barratt did not use exact quantitative methods in their experiments, and as such are desirable, it has been necessary to use a somewhat different technical procedure from that employed by these researchers, but the type of experiment was essentially the same as their's.

### *Production of the Immune Serum, etc.*

The materials used were the red blood cells of the ox, the serum of a rabbit immunised against these, and normal human leucocytes as the phagocytic agents.

The rabbit received intra-peritoneally doses of 10 c.c. of washed ox corpuscles at intervals of a week, 30 c.c. in all being administered before experiments were commenced.

The last injection was made on November 13, 1905. On the 27th of the same month it was found, testing in the usual way, that 0·002 c.c. of the serum produced, when fully complemented, total hæmolysis of 2 c.c. of a 5-per-cent. suspension in normal saline of washed ox corpuscles, after two hours at 37° C. and 12 hours at room temperature.

### *On the Effects of Heat on the Substances in the Serum which Induces Hæmolysis and Phagocytosis.*

The first point to be studied was the influence of heat on the phagocytic action of the serum. With the *undiluted unheated* serum it was found to be a matter of considerable difficulty to perform phagocytic tests owing to hæmolysis somewhat obscuring phagocytosis. With the *undiluted unheated serum* only blood shadows were to be seen in the phagocytes, but on diluting the serum sufficiently to suppress the effects of the complement, hæmolysis was abolished and the red cells could be observed to be phagocytosed, apparently in their normal condition.

In order to find approximately at what degree of dilution hæmolysis would cease to come into play, a series of hæmolytic tests were performed in capillary pipettes. This method was employed in preference to that ordinarily adopted, because with Wright's method of performing phagocytic tests, to deal with absolute quantities is a matter of considerable difficulty.

*Experiment.*—Various dilutions of the *unheated* immune serum were made and equal parts of these dilutions and of a 5-per-cent. suspension of the washed red blood cells of the ox were mixed in a series of capillary pipettes, so that the ultimate proportion of serum in the mixtures varied from 1 in 2 to 1 in 100. These mixtures were placed at 37° C. for two hours. A parallel series was made with serum which had been heated to 55° C. for 15 minutes. This was placed in the same conditions as the former series.

| Dilutions of serum in mixtures. | Result. |
|---|---|
| 1— 2 | Complete hæmolysis. |
| 1— 6 | Complete hæmolysis. |
| 1— 10 | Marked hæmolysis. |
| 1— 20 | Definite hæmolysis. |
| 1— 30 | Trace of hæmolysis. |
| 1— 50 | Trace of hæmolysis. |
| 1— 60 | Hæmolysis absent. |
| 1— 70 | Hæmolysis absent. |
| 1—100 | Hæmolysis absent. |

This experiment shows that in the case of the *unheated* serum no hæmolysis took place in dilutions above 1 in 50 owing to dilution of the native complement and to the fact that no fresh complement was added. With the *heated* serum there was no hæmolysis, even with equal parts of serum and of the suspension of corpuscles, although in such a dilution the *unheated* serum produced complete hæmolysis.

It was therefore decided to begin phagocytic tests with dilutions about 1 in 50 in the case of the unheated serum.

### *Experiment to Show that Heating the Serum to* 55° *C. to* 60° *C. Causes a Diminution of Phagocytosis.*

*Unheated* immune serum was diluted with normal saline solution in the proportions of 1 in 15, 1 in 20, 1 in 30. Of each of these dilutions one part was mixed in a capillary pipette with one part of a 5-per-cent. suspension of washed ox corpuscles and one part of washed human leucocytes, the final dilutions being approximately 1 in 45, 1 in 60, 1 in 90. The tubes were then placed for 15 minutes at 37° C., films being then made and stained with Leishman's stain.

At the same time series were made with portions of the serum which had been heated to 55° C. and 59° C. respectively. The final dilutions in these were 1 in 3, 1 in 6, 1 in 12, 1 in 24, 1 in 45, 1 in 60. A control consisted of one part of 0·85 saline, one part of the suspension of washed ox corpuscles and one part of washed human leucocytes.

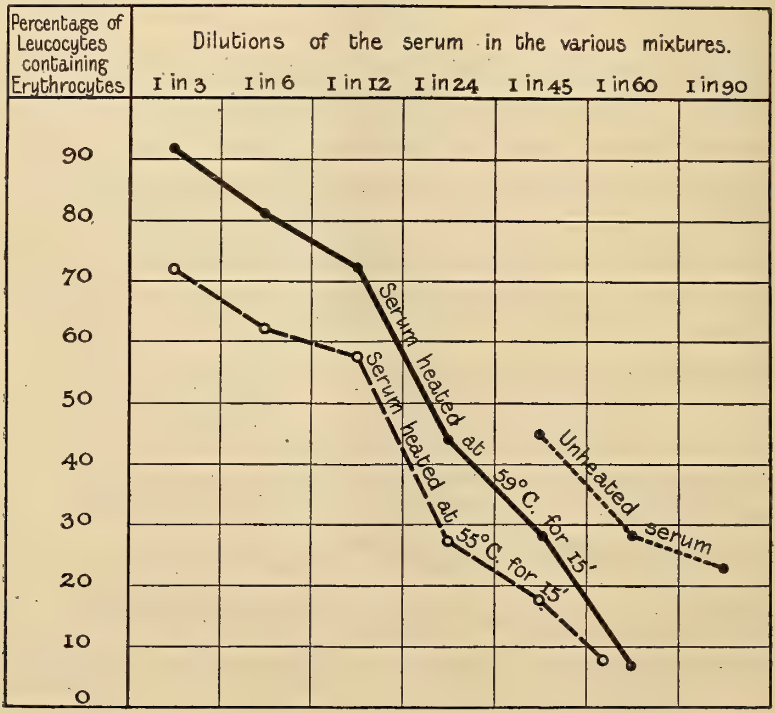
It was found in the first few dilutions that so many red blood cells were taken up by the polymorphonuclear leucocytes, that the individual erythrocytes could not be distinguished and therefore the *percentage* of polymorphonuclear leucocytes containing red blood cells was taken as a criterion of the phagocytic action, 100 leucocytes being counted in each case. Some of the large mononuclear leucocytes contained occasionally one or two red cells, but these were so few as to be insignificant.

It was found that at corresponding dilutions the *unheated* serum produced a considerably greater amount of phagocytosis than did the *heated* and further that it bore greater dilution before giving up this property.

Using the above-mentioned method of enumeration the following results were obtained in the experiment.

| Dilution. | Unheated serum. | Heated at 55° C. | Heated at 59° C. |
|---|---|---|---|
| | Per cent. | Per cent. | Per cent. |
| 1 in 3 | — | 71 | 91 |
| 1 in 6 | — | 61 | 81 |
| 1 in 12 | — | 57 | 72 |
| 1 in 24 | — | 27 | 43 |
| 1 in 45 | 45 | 18 | 28 |
| 1 in 60 | 28 | 8 | 7 |
| 1 in 90 | 23 | — | — |

Saline control = 7 per cent. (The percentages refer to the number of polymorphonuclear leucocytes containing red blood cells.)



These results, which were confirmed by those of subsequent experiments, show that in an immune hæmolytic serum the substance inducing phagocytosis of the appropriate red blood cells is partially destroyed by heating the serum at 55° C. to 60° C. At the time of the experiment 0·002 c.c. of the serum when fully complemented produced complete hæmolysis of 2 c.c. of a 5-per-cent. suspension of washed ox corpuscles after two hours at 37° C. and 12 hours at room temperature.

### *Behaviour of the Hæmolytic Amboceptor towards Heat.*

The next point for investigation was the influence of temperatures similar to those employed in the phagocytic tests, on the hæmolytic amboceptor.

*Experiment.*—Of the immune rabbit's serum two portions were taken, one being left unheated, the other being heated at 55° C. for 15 minutes. Into two series of test-tubes quantities of the serum ranging from 0·01 to 0·0001 c.c. were measured. One series then consisted of heated, the other of unheated serum. All the tubes were equalised in bulk by the addition of 0·85-per-cent. saline solution. To each tube 2 c.c. of a 5-per-cent. suspension of washed ox corpuscles were added with 0·2 c.c. of fresh normal guinea-pig's serum. One control consisted of 2 c.c. of the suspension of red cells with 0·2 c.c. of guinea-pig serum, and another of 2 c.c. of the suspension alone. The tubes were placed at 37° C. for two hours and were subsequently allowed to remain 24 hours at 0° C. The corresponding dilutions in the heated and unheated series showed the same degree of hæmolysis.

### *Result.*

*Unheated Serum.*—Total hæmolysis with all quantities down to 0·005 c.c. Partial hæmolysis with all quantities down to 0·0001 c.c.

*Heated Serum.*—Total hæmolysis with all quantities down to 0·005 c.c. Partial hæmolysis with all quantities down to 0·0001 c.c.

In order to demonstrate conclusively whether there was any appreciable difference between the two series, von Fleischl's hæmometer was employed, the last three corresponding tubes in each series being compared with each other and with the guinea-pig serum control. The tubes were thoroughly shaken up and centrifugalised. The supernatant fluid was then pipetted off, and, if necessary, diluted sufficiently to give a reading between 20 and 60 on the scale before being placed in the chamber of the instrument. The reading found was then multiplied by the amount of the dilution.

The following are the results:—

| | Colour index. |
|---|---|
| Guinea-pig serum control | 64 |
| Unheated serum— | |
| 0·001 c.c. | 440 |
| 0·0005 " | 155 |
| 0·0001 " | 120 |
| Serum heated at 55° C. for 15 mins.— | |
| 0·001 c.c. | 450 |
| 0·0005 " | 220 |
| 0·0001 " | 115 |

It is evident from these numbers that there is practically no difference between the colour indices of the two series; which permits the conclusion to be drawn that the hæmolytic amboceptor is *not* quantitatively diminished when the serum is heated at 55° C. for 15 minutes. Repeated experiments gave exactly similar results and it was found to be a matter of indifference whether the serum was heated *en masse* or in dilution, even in separate small quantities.

This fact is illustrated by the following experiment, which was performed to illustrate at the same time another point, namely, that there may be a large amount of hæmolytic amboceptor present in a *diluted* serum without the coexistence of the body inducing phagocytosis.

*Experiment.*—Four series of tests, A, B, C, D, each consisting of four tubes, were performed. Into successive tubes of each series 0·01, 0·005, 0·003, 0·002 c.c. of the immune serum was placed. The amounts were equalised by 0·85 saline solution. Series A and C were unheated. Series B and D were heated at 55° C. for 15 minutes. To each tube was then added 2 c.c. of a 5-per-cent. suspension of washed ox corpuscles, and to each tube of series A and B 0·2 c.c. of fresh guinea-pig serum (*i.e.*, one unheated, and one heated series was complemented). All the tubes were placed at 37° C. for two hours. It was then found that series A and B showed exactly corresponding degrees of hæmolysis.

| Cubic centimetres. | A. Unheated and complemented. | B. Heated and complemented. |
|---|---|---|
| 0·001 | Almost complete | Almost complete |
| 0·005 | Marked | Marked |
| 0·003 | Slight | Slight |
| 0·002 | Slight | Slight |

This first part of the experiment corroborates the result of the experiment mentioned immediately above.

In series C and D (the proportions of the serum in the mixtures corresponding to 1 in 220, 1 in 450, 1 in 730 and 1 in 1100 approximately), one series being heated and the other unheated, and both being uncomplemented, it was found that there was no sign of hæmolysis when these were compared with the controls, which were the same as in the previous experiment. The tubes of these two series were thoroughly shaken and centrifugalised. The supernatant fluid was pipetted off and the deposits washed thrice with 0·85 saline solution. To each deposit an equal quantity of normal saline was added. They were then well shaken and drawn up and down rapidly in capillary pipettes in order to produce a uniform suspension. Equal parts of each deposit and washed human leucocytes were mixed in capillary pipettes and placed 15 minutes at 37° C., films being then made and stained in the usual manner.

*Result.*—In no case was any phagocytosis observed, although in dilutions of 1 in 10 similarly treated, 93 per cent. of the polymorphonuclear leucocytes contained erythrocytes, which shows that such deposits can be phagocytosed, provided that the substance which induces phagocytosis is present in sufficient amount. Although in series C and D no phagocytosis occurred, yet in dilutions of 1 in 220 hæmolysis was almost complete in the complemented series, which shows that there must have been a large amount of hæmolytic amboceptor present, and that notwithstanding this large amount of amboceptor and an exposure during two hours of the red blood cells to it, no phagocytosis was observed.

This second part of the experiment then shows that in an immune *diluted* hæmolytic serum a considerable amount of hæmolytic amboceptor may be present without rendering the red cells capable of being phagocytosed.

This is supported by observations on non-immune hæmolytic sera. In the case of a guinea-pig's serum which was found in dilutions of 1 in 6 to produce slight hæmolysis of 2 c.c. of a 5-per-cent. suspension of the washed blood corpuscles of a rabbit, it was observed, that in phagocytic tests performed with the unheated serum, the human leucocytes used as the phagocytic agents contained in many cases blood shadows. These were found in 40 to 50 per cent. of the leucocytes in tests performed in the manner described in the former part of this paper. When, however, heated serum is employed no blood shadows are to be seen in the leucocytes nor is there any sign of phagocytosis.

In the case of the serum of an eel it was found that 0·01 c.c. produced after two hours at 37° C. marked hæmolysis of 2 c.c. of a 10-per-cent.

suspension of washed guinea-pig red cells. When heated at 55° C., however, such a serum failed to induce phagocytosis of the red cells after 15 minutes at 37° C., equal parts of the serum, of the suspension of red cells and of washed human leucocytes being employed.

All these facts then tend to show that the hæmolytic amboceptor may be present in a very considerable amount in a serum without giving to the latter the power of inducing phagocytosis of the appropriate red blood cells.

*Conclusions.*—The conclusion naturally come to is that the phagocytosis of red blood cells does not depend on the presence of the hæmolytic amboceptor, since:—

1. The substance which induces phagocytosis is partially destroyed by heat, while the hæmolytic amboceptor is entirely thermostable.

2. The hæmolytic amboceptor may be present in considerable amount in a hæmolytic serum without inducing phagocytosis, notwithstanding prolonged contact of the amboceptor with the red blood cells. This is contrary to the opinion of Savtchenko.*

Dean† has suggested that phagocytosis may be caused by a complement acting through an amboceptor, and that the partial destruction, of the property in the serum inducing phagocytosis, by heat may be due to the destruction of the complement, while the amboceptor, even in the absence of the complement, may still be capable of inducing phagocytosis. This theory, while it is difficult to disprove directly owing to the complement being destroyed at the same temperature as the thermolabile part of the substance inducing phagocytosis, seems to be an improbable one for the following reasons:—

(1) That it is not an action analogous to that of other amboceptors, *e.g.*, that concerned in hæmolysis. If one destroy the complement of a hæmolytic serum by heat, no hæmolysis takes place, notwithstanding the presence of the amboceptor in large amount.

(2) As has been shown above, the hæmolytic amboceptor may be present in large amount in a diluted serum, without that serum having the power of inducing phagocytosis even when Dean's method of testing is employed.

(3) In the dilution experiments recorded above it was shown that one may dilute the complement to such an extent as to abolish hæmolysis, and yet such a serum has a greater "opsonic" power in these dilutions than has the same serum when heated and employed in corresponding dilutions.

If the amboceptor act in the way Dean suggests, it must be supposed to possess, in addition to its complementophilic group, another group which possesses the special function of inducing phagocytosis, *i.e.*, the amboceptor would combine the functions of the second and third receptor types of Ehrlich.

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* *Loc. cit.*, p. 118.

† *Loc. cit.*

The experiments given in this paper, along with those of Barratt,* tend to show, that contrary to the opinion of Dean, Savtchenko was not correct in his conclusion that the specific fixateur, *i.e.*, the hæmolytic amboceptor, induced the phagocytosis of red blood cells, but that on the other hand it is much more probable that this phenomenon is caused by some special body belonging to the class of opsonins.

I have to thank Dr. F. W. Twort for performing the experiments on animals. I have also to express my thanks to Mr. J. A. Craw for suggestions, and to Dr. W. Bulloch of the London Hospital for his kind advice and assistance during the course of my work.

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* *Loc. cit.*

# *Upon the Properties of an Antityphoid Serum obtained from the Goat.*

By Allan Macfadyen, M.D.

(Communicated by Dr. C. J. Martin, F.R.S. Received March 2,—Read March 8, 1906.)

(From the Bacteriological Department, Lister Institute.)

In this communication I propose to give a brief account of the results obtained by the immunisation of the goat with the cell juices of the *Bacillus typhosus*, in continuation of researches already published which had for their main object the production of an antibody for the endotoxin of the typhoid organism.* These experiments met with partial success and failure. It will be sufficient on the present occasion to say that the goat proved a more suitable animal than the horse for arriving at a solution of the theoretical and practical considerations involved. The object was to arrive at the best method of producing with the material employed an antiendotoxin in adequate amount, with a view to its reapplication to the horse.

During the progress of these researches experiments on analogous lines have been published by Dr. Besredka,† and to these I will, in the first instance, refer. Dr. Besredka describes the results following the intravenous injection into a horse of the intact dead and living typhoid bacilli. The injections of the bacilli were carried out for a period of two years. The serum was tested for antiendotoxic properties against (1) killed and dried cultures of the *B. typhosus*, of which 0·01 gramme killed guinea pigs on intraperitoneal injection; (2) a soluble endotoxin extracted from the dead and dried bacilli, of which 1/8 c.c. killed guinea pigs. The results were as follows: 10 or 15 centigrammes of the dried serum (about 1 c.c. and 1·5 c.c. before drying) neutralised 10 or 15 lethal doses of the killed typhoid culture respectively on intraperitoneal injection into the guinea pig, and the 10 centigrammes of the dried serum neutralised 16 lethal doses of the soluble endotoxin. To obtain this low antitoxic value in the horse a period of two years was apparently necessary.

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* "Upon the Immunising Effects of the Intracellular Contents of the Typhoid Bacillus, as obtained by the Disintegration of the Organism at the Temperature of Liquid Air," by Allan Macfadyen, 'Roy. Soc. Proc.,' March 12, 1903; "Upon the Intracellular Constituents of the Typhoid Bacillus," by Allan Macfadyen and Sydney Rowland, 'Centralblatt für Bakteriologie,' Abth. 1, vol. 30, 1901, No. 20; and vol. 34, 1903, Nos. 7 and 8.

† "Etudes sur le bacille typhique et le bacille de Peste," par Dr. Besredka, 'Annales de l'Institut Pasteur,' July 25, 1905: "De l'Antiendotoxine Typhique," par Dr. Besredka, *Ibid.*, February, 1906

The typhoid cell juices employed in my experiments were prepared as follows: Virulent typhoid bacilli were cultivated on nutrient agar in Roux bottles for 18 hours at blood heat. The growth was brushed off and washed with distilled water in a centrifuge for half an hour. The bacilli were then triturated at the temperature of liquid air in the grinding pot already described by Rowland and myself.* The time allowed was 30 minutes per gramme of bacilli. The mass was taken up in 1/1000 solution of caustic potash, and centrifuged for two hours. The supernatant fluid was pipetted off, and represented a 10-per-cent. extract of the ground mass. This was treated with chloroform vapour for half an hour. The juices obtained under these conditions were sterile and toxic on intravenous injection into the test animals employed. The endocellular toxins obtained in this manner are unstable bodies, and the juices rapidly decrease in toxicity. The experiments with kept juices did not indicate that their injection would lead to any marked tolerance for fresh and markedly toxic juices; the probabilities were against this easier and less risky method of procedure. Resort was accordingly made to the use of fresh and acutely toxic juices, which contained on an average 10 to 12 milligrammes of solid matter per cubic centimetre. The fresh juices on intravenous injection were acutely toxic for the goat. The first goat died after the injection of 1 c.c., whilst 1/10 c.c. killed several animals. In two instances 1/20 c.c. killed within 12 hours. Death was preceded by profuse diarrhœa and collapse. Where death did not occur, the injection of 1/20 c.c. was followed by illness and diarrhœa, and 1/30 c.c. rendered certain animals ill, but with less acute symptoms. It was obvious that the intravenous injections would have to be carefully carried out to avoid unduly depressing or killing the animals.

A goat after receiving 1/20 and 1/10 c.c. cell juice died, whilst another, after the injection of doses of 1/20, 1/10, 1/2, and 1 c.c. at intervals of seven days, died within four hours after receiving the last injection. The indication, it appeared to me, was to start with small sublethal doses, and to raise them very gradually at duly spaced intervals. One injection weekly proved to be the safest procedure, and in the later experiments the same dose was repeated until it failed to produce toxic symptoms in the animal. A higher dose was then given and the process repeated. This method proved successful, as the animals became tolerant to otherwise fatal doses of the toxins.

In the experiments here recorded the antiendotoxic action of the serum was tested by intravenous injection in rabbits. The endotoxin likewise killed rabbits acutely, *e.g.*, 1/10 c.c. killed with diarrhœa and collapse, and at times within two hours, whilst 1/20 c.c. was not infrequently a lethal dose. The onset of the toxic symptoms usually occurred in from one to two hours. The conditions were therefore sufficiently stringent in respect of any antitoxic action of the treated goats' serum. I satisfied myself that neither the goats' nor rabbits' serum possessed any appreciable antitoxic power for the toxins employed. Controls were subsequently found to be unnecessary. Three cubic centimetres of normal goat and rabbit serum did not neutralise two or ten lethal doses of the cell juices. The serum of the treated goats was tested against multiple lethal doses of the endotoxin. An estimate was likewise made of the agglutinative and bacteriolytic properties of the serum. I now proceed to an account of the first successful immunising experiment.

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* 'Centralblatt f. Bakter.,' vol. 34, No. 7, 1903.

*Billy Goat I.*—Received following intravenous injections of toxic cell juices of *B. typhosus* :—

| 1905. | | | | | 1905. | | | | |
|---|---|---|---|---|---|---|---|---|---|
| May | 16 ...... | 1/20 | c.c. | Ill. | July | 7 ...... | 1 | c.c. | No symptoms. |
| ,, | 24 ...... | 1/10 | ,, | Ill. | ,, | 14 ...... | 1·5 | ,, | No symptoms. |
| June | 2 ...... | 1/5 | ,, | Ill. | ,, | 21 ...... | 1·5 | ,, | No symptoms. |
| ,, | 9 ...... | 1/2 | ,, | Ill. | ,, | 28 ...... | 2 | ,, | Ill. |
| ,, | 16 ...... | 1 | ,, | Ill. | Aug. | 4 ...... | 2 | ,, | No symptoms. |
| ,, | 23 ...... | 1 | ,, | Ill. | ,, | 11 ...... | 2·5 | ,, | Dead next day. |
| ,, | 30 ...... | 1 | ,, | Ill. | | | | | |

It was evident that with succeeding animals an even more careful system of dosage would have to be adopted. The goat was bled at intervals and its serum tested. In all instances the serum-toxin mixture was kept at 37° C. for 30 minutes previous to intravenous injection in the rabbit. The results are given in Table II.

### I.—Test of Normal Goat's Serum.

| | | | | |
|---|---|---|---|---|
| Rabbit | I ......... | 3 c.c. serum + 1 c.c. toxin. | Dead. |
| ,, | II ......... | 2 ,, + 1 ,, ,, | Dead. |
| ,, | III ......... | Control. 1/10 ,, ,, | Dead. |
| ,, | IV ......... | ,, 1/20 ,, ,, | Diarrhœa. |

Three cubic centimetres of normal serum did not neutralise 1 c.c. of a toxic cell juice of which 1/10 killed and 1/20 c.c. produced acute diarrhœa in the rabbit.

II.—Tests of Serum of Goat I.

| Date. | Test animal. | Amounts injected. | Results. |
|---|---|---|---|
| June 23 | Rabbit 1 | 3 c.c. serum + 1 c.c. toxin. | Alive. |
| ,, | ,, 2 | Control, 1 c.c. toxin. | Dead. |
| July 7 | ,, 1 | 3 c.c. serum + 2 c.c. toxin. | Alive. |
| ,, | ,, 2 | 3 ,, +1 ,, | ,, |
| ,, | ,, 3 | 2 ,, +1 ,, | ,, |
| ,, | ,, 4 | Control, 2 c.c. toxin. | Dead, 4 hours. |
| ,, | ,, 5 | ,, 1/20 ,, | ,, 18 ,, |
| July 14 | ,, 1 | 3 c.c. serum + 2 c.c. toxin. | Alive. |
| ,, | ,, 2 | 2 ,, +2 ,, | ,, |
| ,, | ,, 3 | 1 ,, +1 ,, | ,, |
| ,, | ,, 4 | 1/10 ,, +1 ,, | ,, |
| ,, | ,, 5 | Control, 1/10 c.c. toxin. | Dead. |
| ,, | ,, 6 | ,, 1/20 ,, | Diarrhœa and collapse. |
| July 21 | ,, 1 | 1 c.c. serum + 1 c.c. toxin. | Alive. |
| ,, | ,, 2 | 1/2 ,, +1 ,, | ,, |
| ,, | ,, 3 | 1/10 ,, +1 ,, | ,, |
| ,, | ,, 4 | Control, 1 c.c. toxin. | Dead. |
| ,, | ,, 5 | ,, 1/10 ,, | Diarrhœa and collapse. |
| ,, | ,, 6 | ,, 1/15 ,, | ,, ,, ,, |
| August 4 | ,, 1 | 1/20 c.c. serum + 1 c.c. toxin. | Alive. |
| ,, | ,, 2 | 1/50 ,, +1 ,, | ,, |
| ,, | ,, 3 | 1/100 ,, +1 ,, | ,, |
|  |  | Control not made. |  |
| August 11 | ,, 1 | 1/20 c.c. serum + 1 c.c. toxin. | ,, |
| ,, | ,, 2 | 1/50 ,, +1 ,, | ,, |
| ,, | ,, 3 | 1/100 ,, +1 ,, | Dead. |
| ,, | ,, 4 | Control, 1 c.c. toxin. | ,, 2 hours. |
| ,, | ,, 5 | ,, 3/10 ,, | ,, 18 ,, |
| ,, | ,, 6 | ,, 1/10 ,, | ,, 18 ,, |

The death of the goat prevented further injections and tests being made. It will be seen that demonstrable antiendotoxins had developed in the goat and that the results were encouraging. After 12 injections of typhoid cell juice, 1/50 c.c. of the goat's serum protected a rabbit against 10 lethal doses of the typhoid endotoxin. This property was not present in 3 c.c. of normal goat's serum. It is not my intention in this paper to refer in detail to the production of agglutinins and bacteriolysins. It will be sufficient to state that whilst the amount of agglutinins present in the serum varied, the highest titrate obtained was in a dilution of 1/1,000,000. After 12 injections of typhoid cell juice, 1/10000 c.c. of the goat's serum protected guinea-pigs on intraperitoneal injection against 10 lethal doses of the *B. typhosus.* The serum was, therefore, proved to possess marked antiendotoxic, agglutinative and bacteriolytic properties.

The next step was to control these results by the immunisation of fresh goats, and to carry out the process in a still more careful manner. The method adopted is given in the following schedule :—

*Nanny Goat II.*—Received following intravenous injections of toxic cell juices of *B. typhosus*—

| | | |
|---|---|---|
| 1905. | | |
| Oct. 13 ...... | 1/20 c.c. | Ill. |
| „ 20 ...... | 1/20 „ | No symptoms. |
| „ 27 ...... | 1/15 „ | Ill. |
| Nov. 3 ...... | 1/15 „ | No symptoms. |
| „ 10 ...... | 1/10 „ | No symptoms. |
| „ 17 ...... | 1/10 „ | Ill. |
| „ 24 ...... | 1/10 „ | Ill. |
| Dec. 1 ...... | 1/10 „ | No symptoms. |
| „ 8 ...... | 1/8 „ | Ill. |
| „ 15 ...... | 1/8 „ | No symptoms. |
| 1905. | | |
| Dec. 22 ...... | 1/8 c.c. | No symptoms. |
| „ 29 ...... | 1/6 „ | Ill. |
| 1906. | | |
| Jan. 5 ...... | 1/3 „ | No symptoms. |
| „ 12 ...... | 1/6 „ | No symptoms. |
| „ 19 ...... | 1/4 „ | No symptoms. |
| „ 26 ...... | 1/4 „ | No symptoms. |
| Feb. 2 ...... | 1/3 „ | Ill. |
| „ 9 ...... | 1/3 „ | No symptoms. |

## III.—Tests of Serum of Goat II.

| Date. | Test animal. | Amounts injected. | Results. |
|---|---|---|---|
| December 29 ...... | Rabbit 1 | 1/20 c.c. serum + 1 c.c. toxin. | Alive. |
| „ ...... | „ 2 | 1/50 „ + 1 „ | Dead. |
| „ ...... | „ 3 | 1/100 „ + 1 „ | Alive. |
| „ ...... | „ 4 | Control, 1/5 c.c. toxin. | Dead, 2 hours. |
| „ ...... | „ 5 | „ 1/10 „ | Diarrhœa and collapse. |
| January 19, 1906.. | „ 1 | 1/50 c.c. serum + 1 c.c. toxin. | Alive. |
| „ ... | „ 2 | 1/100 „ + 1 „ | Dead. |
| „ ... | „ 3 | Control, 1/10 c.c. toxin. | „ |
| January 26 ......... | „ 1 | 1/50 c.c. serum + 1 c.c. toxin. | Alive. |
| „ ......... | „ 2 | 1/100 „ + 1 „ | Dead. |
| „ ......... | „ 3 | Control, 1/10 c.c. toxin. | „ |
| February 2 ......... | „ 1 | 1/10 c.c. serum + 1 c.c. toxin. | Alive. |
| „ ......... | „ 2 | 1/20 „ + 1 „ | „ |
| „ ......... | „ 3 | 1/50 „ + 1 „ | „ |
| „ ......... | „ 4 | 1/50 „ + 1/2 „ | „ |
| „ ......... | „ 5 | Control, 1/10 c.c. toxin. | Dead, 2½ hours. |
| „ ......... | „ 6 | „ 1/20 „ | „ 2 „ |
| „ ......... | „ 7 | „ 1/30 „ | „ 18 „ |
| February 9 ......... | „ 1 | 1/50 c.c. serum + 1 c.c. toxin. | Alive. |
| „ ......... | „ 2 | 1/50 „ + 1 „ | „ |
| „ ......... | „ 3 | 1/100 „ + 1 „ | Dead. |
| „ ......... | „ 4 | 1/100 „ + 1/2 „ | Alive. |
| „ ......... | „ 5 | Control, 1/10 c.c. toxin. | Dead. |
| „ ......... | „ 6 | „ 1/20 „ | Diarrhœa and collapse. |

Three goats are in process of immunisation by this method of small and gentle dosage and have not up to the present succumbed. It will be observed that whilst in the case of Goat I the dosage was raised from

1/20 to 2½ c.c., the dose reached in Goat II when the final test was made only amounted to 1/3 c.c. of juices containing 10 to 12 milligrammes of solid matter. The figures demonstrate the production in considerable amount of an antiendotoxin. The highest titrate obtained was a neutralisation of 30 ascertained lethal doses of endotoxin by 1/50 c.c. of the serum of Goat II. The results confirmed those obtained in the case of Goat I, and were equivalent despite the injection of a much smaller gross amount of typhoid cell juice. The raising of the antiendotoxic value of the serum had likewise been accomplished without any serious disturbance in the health of the animal. This was the difficulty which had retarded the progress of the initial experiments.

Some tests were made as to the action of the serum on the endotoxin when each was injected separately. The results were as follows:—

*Rabbit I.*—Fifteen lethal doses of typhoid cell juice were injected into a vein of the right ear and 1 c.c. serum into a vein of the left ear. A second injection of 1 c.c. serum was given 20 minutes later. The animal survived.

*Rabbit II.*—Received five lethal doses of the toxic juice in the right ear and 1 c.c. serum in the left ear. The animal survived.

*Rabbit III.*—Received five lethal doses intravenous and ¾ hour later at the onset of toxic systems 2 c.c. of serum. The rabbit survived. The serum, therefore, acted on separate injection into the blood stream.

The serum of Goat II was also tested against the endotoxin of the cholera organism. One-half cubic centimetre of typhoid serum was added to three lethal doses of cholera cell juice, and the mixture, after incubation for 30 minutes at blood heat, was injected into a rabbit. The animal died 2½ hours after the injection. One-half cubic centimetre of a typhoid serum, which had been found to protect against 30 lethal doses of typhoid endotoxin, did not protect a rabbit against three doses of cholera endotoxin, and was to this extent specific. The agglutinative power of the serum rose to 1/1,000,000, and 1/10000 c.c. protected the guinea-pig against 10 lethal doses of the typhoid bacillus.

The serum was finally tested in dilutions of 1/10, 1/100, and 1/500 c.c. for any evidence of a precipitin reaction on the fresh typhoid cell juices. The result was negative. There had been no appreciable development of precipitins in a serum containing at the time, when it was tested, marked antiendotoxic properties.

## *Conclusions.*

1. The intravenous injection of the goat with the toxic cell juices of the *B. typhosus* (obtained under the conditions described) in small and carefully regulated doses resulted in the production of an antiendotoxin.

2. The antiendotoxic value, as so far tested, reached a point at which 1/50 c.c. of the serum neutralised 30 lethal doses of the toxic typhoid cell juice. This action was not demonstrable in 3 c.c. of normal goat's serum, and was obtained after about four months' treatment of the goat. The results, after a more rapid method of immunisation, are better *qua* goat and rabbit than those obtained by Dr. Besredka in the course of two years with dead and living bacilli *qua* horse and guinea-pig.

3. The serum was also agglutinative for the *B. typhosus,* the titrate rising to 1/1,000,000.

4. The serum was also bacteriolytic, 1/10000 c.c. neutralising 10 lethal doses of the *B. typhosus.*

5. The serum did not give a precipitin reaction with typhoid cell juices.

6. The serum whilst neutralising the typhoid did not neutralise the cholera endotoxin.

My next step will be to test in how far it is possible to obtain analogous results in the horse.

Analogous results have been obtained indicating the production of an antibody for the endotoxin of the cholera organism.

I am greatly indebted to Mr. E. T. Thompson for invaluable aid as well as for an important modification which has rendered the grinding process void of danger.

# *On the Synapsis in Amphibia.*

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(Communicated by J. Bretland Farmer, F.R.S., F.L.S. Received December 5, 1905,—Read January 18, 1906.)

[PLATES 20—23.]

In 1903 and 1904 one of us, in conjunction with Professor Farmer, described the maiotic process in a variety of animals and plants.* From the observations then accumulated it was in the first place shown that what we termed the maiotic process appears to be the same throughout the animal and vegetable kingdoms. In the second it was pointed out that the general scheme we were able to formulate was in accord with the particular description of the metamorphosis given by Korschelt† for Ophryotrocha as long ago as 1895, as well as with the account of the same change in some amphibia given by Montgomery‡ in the same year as ourselves.

According to this conception of the maturation process, the reduction in the number of chromosomes to one half is brought about by a pairing of somatic chromosomes which takes place in the prophase of the first maiotic (heterotype) divisions. In this way we have in some mammals, for example, 16 pairs of chromosomes in the place of 32 single elements.

These chromatic *gemini*, as we propose to call them, go on to the spindle in the same way as ordinary premaiotic or somatic chromosomes. But during the division each of the respective gemini separate into the two component parts; so that in the cases of mammals above referred to there are 16 premaiotic chromosomes distributed to each daughter cell.

According to this view it would appear that during the first maiotic division no longitudinal fission of the chromosomes composing the gemini comes into play, and the longitudinal split which is visible in the spirem figure only effects that incomplete fission of the daughter elements first observed by Flemming in the diasters of the first maiotic division in amphibia (see fig. 22).

In this way the longitudinal split of the thread which takes place in the spirem stage only becomes completed and effective during the second maiotic (homotype) division. Consequently these two divisions with their respective prophases form a stage in the cell cycle of plants and animals which differs entirely from the divisional sequence before and after it.

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* *Cf.* Farmer and Moore, 'Roy. Soc. Proc.,' May, 1903; Farmer and Moore, 'Quart. Journ. Micros. Sci.,' vol. 48; Farmer and Shove, 'Quart. Journ. Micros. Sci.,' vol. 48; Moore and Robinson, 'Quart. Journ. Micros. Sci.,' vol. 48.

† Korschelt, 'Zeitschr. für Wiss. Zool.,' vol. 9.

‡ Montgomery, 'Biol. Bull.,' vol. 4, 1903.

The present communication deals with the prophase of the first maiotic division in Triton, and with the subsequent division up to the appearance of the diaster. As we have worked out the stages in this animal in great detail, we propose to give the results of our observations without discussion in the text, but to indicate by means of footnotes those points in which the fresh observations differ from the existing accounts of the maturation process in general, and of the same phenomena among amphibia in particular.

It may however be pointed out that the results of this investigation have been entirely to confirm the general interpretation of the maiotic metamorphosis already published by Professor Farmer and one of ourselves.*

At the same time it should be noted from the beginning that this view of the maiotic process entails a complete revision of the older conceptions of the nature of the first maiotic (heterotype) division among the higher vertebrata, such as those embodied in the able works of Flemming, Meves, and many others.

During the early summer (June) in England the testes in Triton are found to present all the phases in the cell cycle, from premaiotic cells dividing in their characteristic manner, to the spermatids that have been produced by the second maiotic (homotype) division (see fig. 1, Plate 20).

From this figure it will be seen that in the section of the testes which the drawing represents the spermatogenesis is proceeding from the top towards the bottom of the Plate. Immediately below the peritoneal attachments groups of cells are seen, some with their nuclei at rest, others dividing; while in the region marked (*c*) nuclei are seen as we pass downwards, which are at first indistinguishable from those in (*b*), but which gradually alter in the appearance until we reach the lower part of (*c*).

The division figures encountered in region (*b*) are all of the ordinary premaiotic type, and the appearance which comes over the resting nuclei in (*c*) as we pass downwards is the appearance produced by the advent of the synaptic metamorphosis in Triton when seen under a low power. Still lower than the region (*c*) the two maiotic divisions (heterotype and homotype) are encountered together.

The above will serve to orientate the original and relative positions of the cells which have been taken as individual illustrations of the successive phases of the synaptic change. But before leaving the small scale figure, it may be noted that upon examination the resting nuclei in region (*b*) differ from those in the (*c*) area in that they obviously contain more of the apparently nucleola bodies scattered within the membrane.

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* Farmer and Moore, *loc. cit.*

When resting cells of the region (*b*) are examined under a high power, it is seen that nucleolus-like bodies visible under a lower power resolve themselves into the short chromatic rods represented in figs. 2 and 3.

These bodies are seen to lie more or less parallel to one another within the cells, so that the appearance of the nuclei varies from that given in fig. 2, where the chromatic rods are seen from one side, to that represented in fig. 3, where the same rods are looked at from one end.

The definite nature of these bodies is sufficiently obvious from the figures, but their significance only became apparent upon ascertaining that their number is always about 24; that is to say, it was found, after counting in about 50 individual cells, that the number 24 was obtained for the great majority, while in the few cases in which it was above or below this figure such divergence was limited to one or two, and in nearly all these exceptions it was possible to attribute the divergence to some optical difficulty. The bodies lay over one another in the line of vision, or were hidden and confused by the nuclear membrane and the like.

The premaiotic resting cells in the testes of Triton are found then to contain in their nuclei chromatic structures which at first might be, and generally have been, regarded as "chromatin nucleoli" (Flemming); but they correspond in numbers to the chromosomes of the premaiotic prophases and division figures, for in this animal the number of the premaiotic chromosomes is 24. These bodies correspond in fact exactly to what in 1904 we have already described as the Anlagen of the premaiotic chromosomes in the corresponding cells in the testes of Periplaneta,* and there can be no doubt that they represent also the structures subsequently alluded to as prochromosomes by Overton,† Miyake,‡ and Strasburger§ in the same stage in certain mono- and dicotyledonous plants.

In Triton these structures are not restricted to the premaiotic cells of the testis, but are equally apparent and have the same relationships in other tissues of the animal's body.

In some respects the bodies in question are more definite and stainable in the resting premaiotic cells of Triton than they are in the corresponding elements of Periplaneta, and in both cases their subsequent history during the premaiotic divisions is perfectly simple. In Triton each Anlagen (or prochromosome) becomes gradually enlarged and thickened into the long premaiotic chromosomes of the spirem figure, while in Periplaneta they gradually assume the form of dense short rods characteristic of the premaiotic division figures of that arthropod.

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* Farmer and Moore, 'Quart. Journ. Microsc. Sci.,' vol. 48, *loc. cit.*, Plate 38 and text.

† Overton, J. B., 'Über Reduktionsteilung in dem Pollenmutterzellen einiger Dikotylen.'

‡ Miyake, K., 'Über Reduktionsteilung in dem Pollenmutterzellen einiger Monokotylen.'

§ Strasburger, E., "Typische und Allotypische Kernteilung," 'Jahrb. für Wiss. Botanik,' vol. 42.

In both cases the chief interest of these bodies lies in the fact that they obviously represent the chromosomes of division during rest; and we may say without reserve that their presence at all stages of rest between the successive premaiotic divisions seems to conclusively prove the permanence of the chromosomes from one cell generation to another.

Examination of the resting maiotic nuclei given in figs. 1 and 2 shows that, so far as can be seen, the Anlagen consist of irregular rods composed chiefly of chromatin, and either suspended in a fine linin meshwork within the nucleus or attached to the nuclear membrane, where the chromatin is seen to be spread out, and to give to the membrane itself the peculiar thickened appearance characteristic of the stage. No other structures with the exception of irregular nucleoli are visible within the resting premaiotic nuclei.

Before the maiotic metamorphosis sets in the Anlagen are seen to be single and discrete, figs. 2, 3; but as we pass to an examination of the cells in the later phases in the region *c*, where the synaptic change is gradually proceeding towards the formation of the coarse spirem, we find that the Anlagen are no longer single, in fact, the diminution in the number of what, under a low power would be taken for nucleoli is seen to be produced by a pairing of the bodies while the nuclei themselves still remain at rest.

The appearance which such cells present is represented in figs. 1, 4, and 5. Fig. 4 shows a cell in this condition in the same relative position as that represented in fig. 2. The associations produced in this way (which we propose to call *gemini*) are again represented in fig. 5, where the gemini are seen end on, as were the single Anlagen in fig. 3.

We have then in the testis of Triton premaiotic cells which, like the premaiotic or somatic cells of rest of the animal's body, possess structures that represent during rest the individual chromosomes of the successive division figures, and these bodies during the first phase of the synaptic metamorphosis that is perceptible, conjugate or pair so as to form 12 gemini in the place of the 24 single Anlagen (prochromosomes).

When the gemini have been produced it is seen, as in fig. 4, that they are related in a conspicuous manner by shreds and strings of linin to chromatin patches on the membrane of the nucleus; and there are indications that before the gemini are formed the linin of each single chromosome extends out to the limits of the nucleus.

At all events, when the gemini have been produced each chromatic rod rapidly extends outwards along linin threads in the manner represented by fig. 6. In this we soon get an appearance which would be quite easy to misinterpret as a fission in a spirem were not the earlier stages of the process present in the surrounding tissue.

A somewhat later stage is represented in fig. 7, where it is seen that the original components of the gemini are rapidly elongating out into loops or polarised chromatic bands. Cells possessing the characteristics represented in figs. 6 and 7, constitute, in fact, the only representative in Triton of the strong synaptic contraction figure so conspicuous in many other forms of animals and plants. At a later stage the gemini, although still clearly visible, have so far elongated and moved from their original positions as to give the appearance represented in fig. 9.

This movement, which corresponds to the unwinding of the synaptic loops in mammals and other forms, is seen to have reached a further stage in fig. 8. Here the condition of the early coarse spirem stage is clearly foreshadowed, and by stages such as those represented in figs. 10 and 11 the characteristic polarised loops of the late spirem figure become gradually formed.

It appears then that the polarised loops are produced by the growth and elongation of the original gemini, and that consequently each loop represents two premaiotic chromosomes, which may be associated together at one, or both, of their ends. In general, in Triton and elsewhere the chromosomes forming the loops remain connected together only by the ends which originally lay towards the nuclear interior, the outer pair of ends abutting upon some portion of the nuclear membrane, and always in the late stages of the spirem figure becoming widely detached from each other.

Stages in the formation of the individual loops and the mode of attachment of the chromosomes together at the round ends of the loops can be seen in figs. 9, 10, 11, and 12.

From the original formation of the gemini until the production of the spirem loops, figs. 10 and 11, the chromatin in each lateral component or chromosome is seen to be in the form of irregular granules, and remains scattered along a linin framework; but at subsequent stages (figs. 12, 13, and 14) these chromatin particles become arranged, or split as it were, into two longitudinal rows, and the loops from this time onwards present the characteristic split appearance represented in figs. 13, 14, 15, and 16. In stages such as that given in fig. 14 every loop is completely divided throughout its entire length, and the longitudinal halves of the thread may divaricate from each other as far as is to be seen in portions of the loops represented in fig. 15. At this stage, and after, the loops are very long indeed, stretching in some cases completely round the whole nucleus, and it is, we think, without doubt chiefly owing to this circumstance that their history has been in general misinterpreted.*

A comparison of figs. 14, 15, and 16 will show that the split after becoming, as in fig. 15, very conspicuous, gradually closes up again, fig. 16, whilst in figs. 17 and 18 the closing process is still further completed. Even in fig. 18, however, the split is in places still visible, and at fig. 19, when the loops have been finally resolved into the adult gemini of the first maiotic division figure, the fission is not in all cases completely lost.

In other animals at the same stage, Periplaneta for example, the fission, as has been shown in a former work,† is often visible throughout the whole spindle figure of the first maiotic mitosis, and in such cases the origin of the fission of the daughter chromosomes is obvious.

In the final stages of the prophase in Triton (figs. 18 and 19) the gemini assume the various forms characteristic of the first maiotic division, and during the diaster the rings and loops break apart in the manner represented in figs. 21 and 22.

As soon as the diastral V's have been formed the original longitudinal split becomes here also again clearly visible (fig. 22). It is moreover quite easy to show in Triton as in other cases that it is this fission which functions in the final maiotic (homotype) division.

To recapitulate (see diagram):—In Triton it is found, when sufficient

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* As is well known, Flemming, and after him many others, originally regarded the split of the spirem as opening out in lengths to form the rings and loops presented by the adult so-called heterotype chromosomes, and the longitudinal fissions of the daughter elements in the diaster of this division as a subsequent and independent fission. Dixon (‘Roy. Ir. Acad. Proc.,’ vol. 3) regarded the split seen in the spirem as due to an approximation similar to that witnessed in the formation of the gemini. Berg (‘La Cellule,’ vol. 21), Overton, and Miyake (*loc. cit.*) have adopted a similar view, regarding the late split in the spirem as a lingering expression of the conjugation during the formation of the several gemini. At times a similar view has been taken by Strasburger (*loc. cit.*) and others. We regard our present observations, as well as those upon numerous other forms dealt with in our former paper with Professor Farmer, as incompatible with the idea contained in the works of the above authors, namely, that the split in the spirem of the first maiotic division is due to an approximation, and are inclined to think that this view can only have originated through a confusion having been made between the conjugation during the formation of the gemini and the longitudinal fission which, without any doubt whatever, does take place during the spirem stage.

Montgomery (‘Biol. Bull.,’ vol. 4), in the same year as ourselves (Farmer and Moore, ‘Roy. Soc. Proc.,’ 1903, *loc. cit.*), regarded the split in the spirem in Amphibian heterotype prophases as not constituting the opening of the loop or ring of the spindle figure, and the fission of the daughter diastral elements as due to the split visible in the spirem.

† Farmer and Moore, ‘Quart. Journ. Micros. Sci.,’ vol. 48, *loc. cit.*

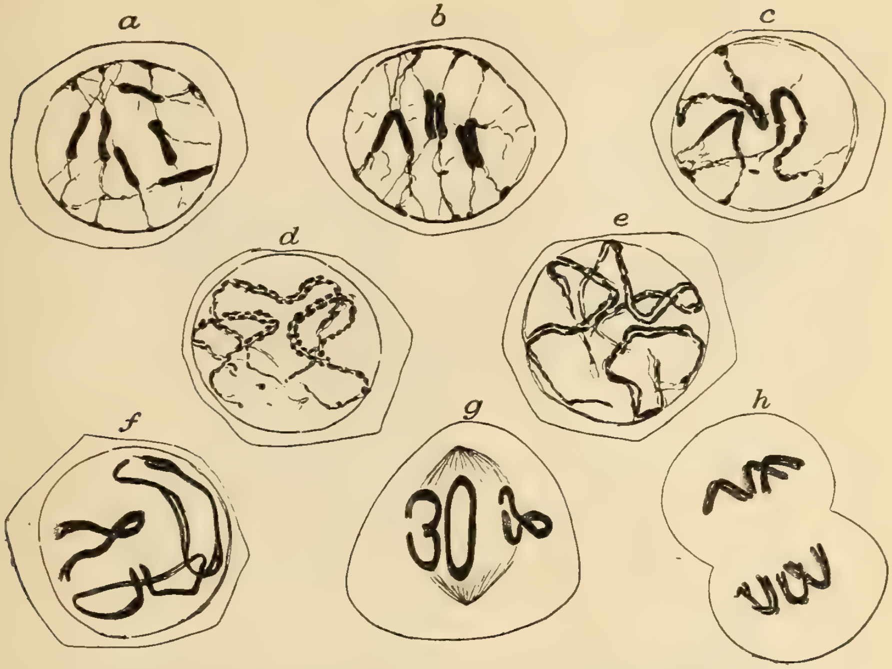

Diagram representing the course of the synaptic metamorphosis. For the sake of clearness only six chromosomes are represented in the premaiotic resting cell (*a*). At (*b*) the chromosomes are uniting; (*c*) shows the conversion of the gemini into the young loops. At (*d*) is seen the split in the chromosomes; (*e*) shows a later stage; (*f*) shows the transformation of the loops into the adult gemini (heterotype chromosomes); (*g*) represents their appearance on the spindle; (*h*) shows the diastral chromosomes, three in each cell, with the longitudinal split begun in the preceding prophase.

optical power and efficient preservation are combined, that: The somatic chromosomes are visible in the resting cells; that during the inception of the synaptic phase these chromosomes pair so as to form double bodies which are the forerunners of the adult gemini (heterotype chromosomes, allotype chromosomes, bivalent chromosomes, etc.); that by growth and elongation the gemini constitute the polarised loops of the first maiotic prophase; that these loops become longitudinally split, and later each longitudinally fissed aggregate rolls itself up into one or other of the forms assumed by the adult gemini; that in these later stages in Triton the longitudinal fission of the chromosomes becomes almost, but not quite closed up, and in the diaster the separated chromosomes again exhibit it; while finally it is seen that this split functions in the second maiotic (homotype) division.

## DESCRIPTION OF PLATES.

[Figs. 2—22 are drawn with a 3-mm. long-tube Zeiss apochromatic 1·40 aperture, and a 27 ocular.]

### PLATE 20.

FIG. 1.—Section of testis of *Triton* (June) as seen under a low power lens. At the upper margin is the attachment between the testis and the body wall and the membrane which surrounds the tubules. The foot-cells are not shown. Near the upper margin to the right are two "male ova"; towards the left are tubules with cells in the premaiotic (somatic) stage dividing at (*b*). The region marked (*b*) contains the zone of transformation wherein the synaptic gemini are constituted. Late synaptic (heterotype) prophases are seen at (*c*), and the first and second maiotic divisions lower down.

FIG. 2.—Cell from the upper premaiotic region, the nucleus showing bodies representing the 24 chromosomes while in a condition of complete rest.

FIG. 3.—Cell from the same region as fig. 2, viewed from a direction at right angles to the foregoing; the bodies representing chromosomes seen end on.

FIG. 4.—Cell in the first phase of the synapsis, showing the bodies representing the resting premaiotic chromosomes uniting in pairs to form the gemini.

### PLATE 21.

FIG. 5.—Cell from the same region as fig. 4, viewed from a direction at right angles showing the gemini end on.

FIG. 6.—Cell advancing in the synapsis, the gemini elongating.

FIG. 7.—Synapsis further advanced, the gemini elongating into loops.

FIG. 8.—Synapsis still further advanced, the gemini moving from their original central position.

FIG. 9.—The gemini becoming converted into loops.

FIG. 10.—The gemini becoming polarised into the loops of the coarse spirem figure.

### PLATE 22.

FIG. 11.—Cell showing the coarse spirem, the threads not split.

FIG. 12.—Same as fig. 11, but showing the first traces of the longitudinal split.

FIGS. 13, 14.—Longitudinal split more apparent.

FIG. 15.—The split seen at its maximum.

FIG. 16.—The split seen closing up at places.

### PLATE 23.

FIGS. 17, 18.—Cells showing the ends of the loops with portions of the split closed up, others open.

FIGS. 19, 20, 21.—Stages in the first maiotic division.

FIG. 22.—The diaster of the first maiotic division, showing the split in the diastral chromosomes.

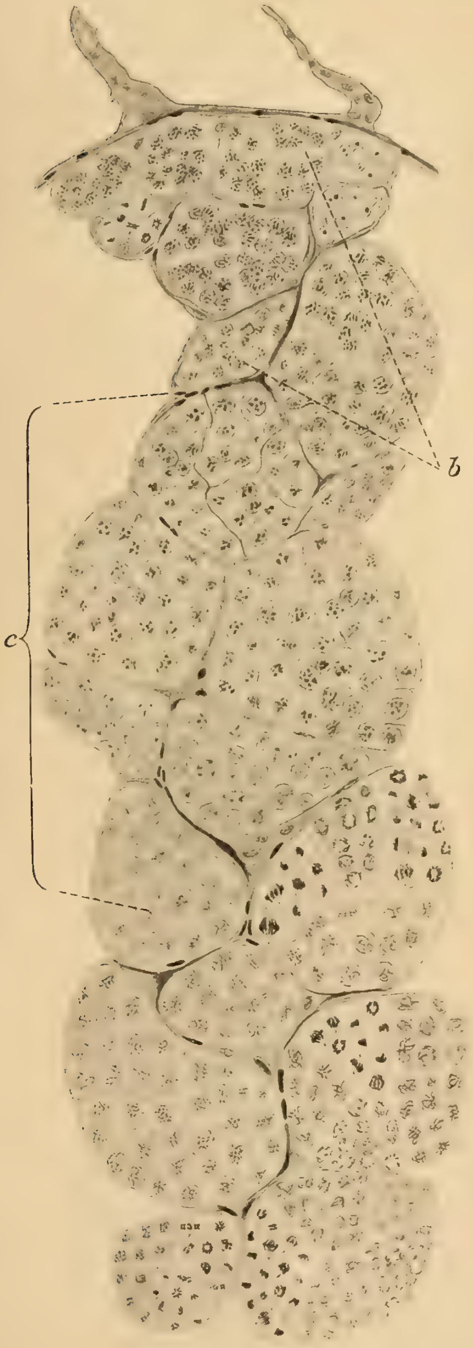

FIG. 1.

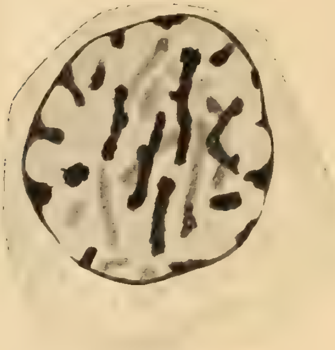

FIG. 2.

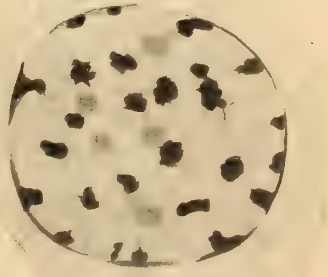

FIG. 3.

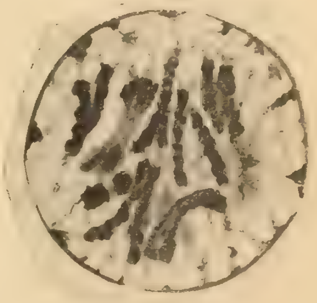

FIG. 4.

*Moore and Embleton.* *Roy. Soc. Proc., B. vol. 77, Plate* 21.

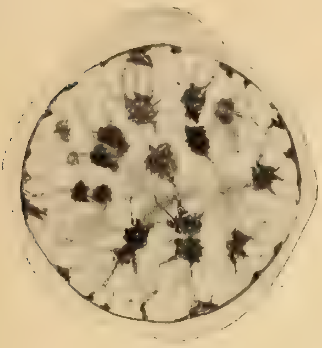

Fig. 5.

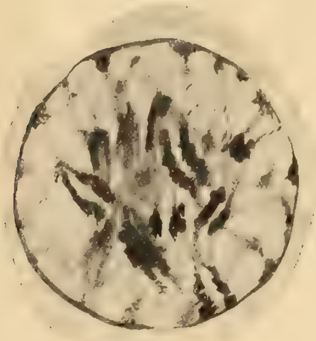

Fig. 6.

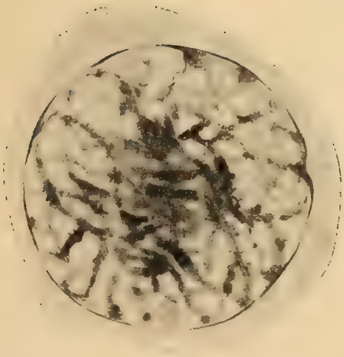

Fig. 7.

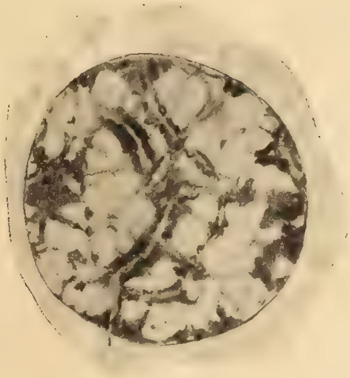

Fig. 8.

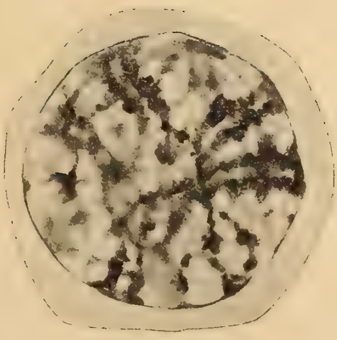

Fig. 9.

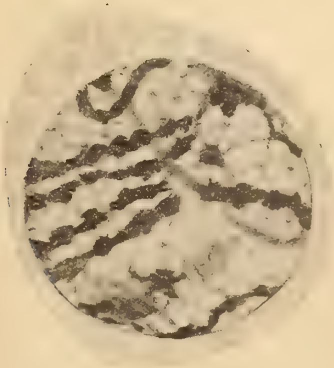

Fig. 10.

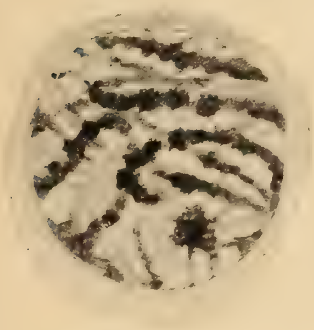

FIG. 11.

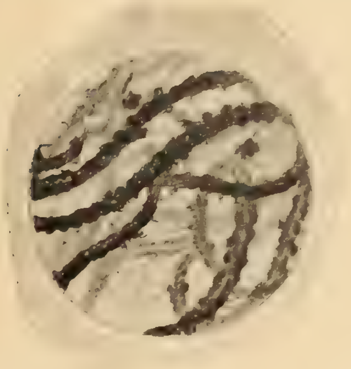

FIG. 12.

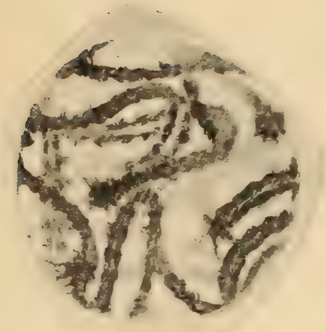

FIG. 13.

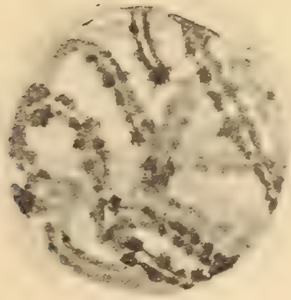

FIG. 14.

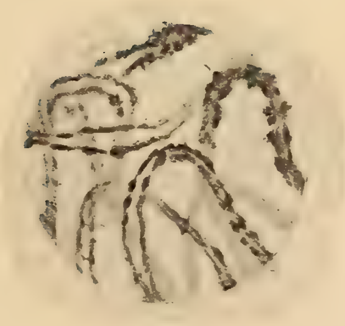

FIG. 15.

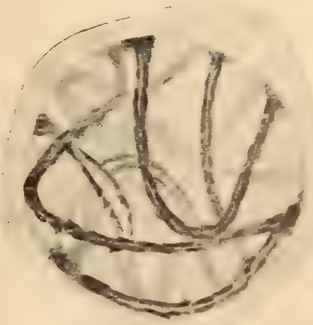

FIG. 16.

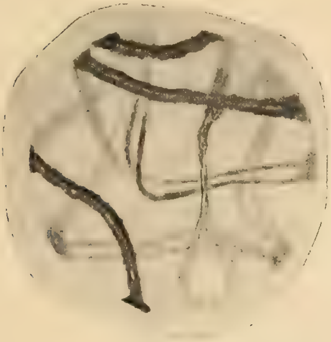

FIG. 17.

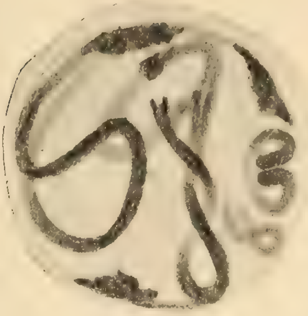

FIG. 18.

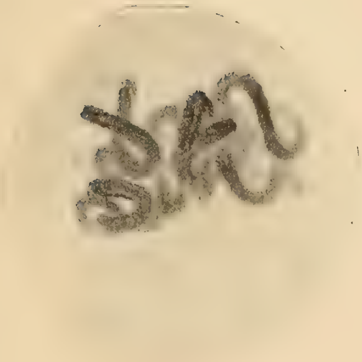

FIG. 19.

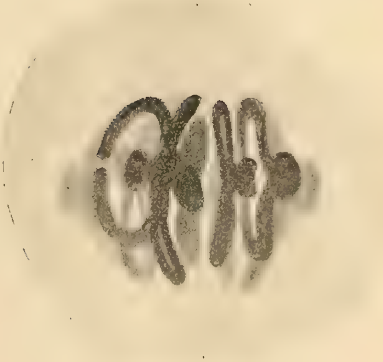

FIG. 20.

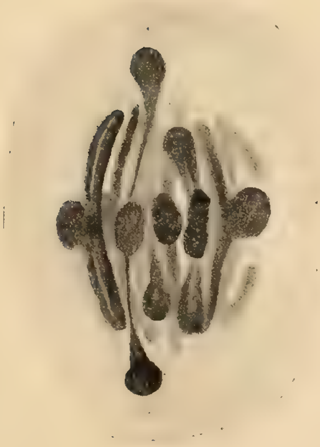

FIG. 21.

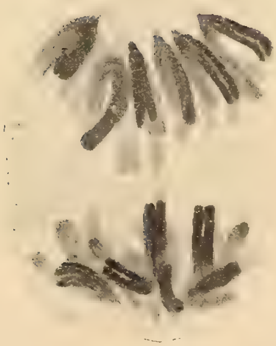

FIG. 22.

# *On the Existence of Permanent Forms among the Chromosomes of the First Maiotic Division in Certain Animals.*[*]

By J. E. S. Moore, A.R.C.S., F.L.S., Director of the Cancer Research Laboratories, University of Liverpool, and George Arnold.

(Communicated by J. Bretland Farmer, F.R.S. Received December 13, 1905,—
Read January 18, 1906.)

[Plates 24 and 25.]

Many cytological investigators have drawn attention to the peculiar forms assumed by the heterotypical aggregates of chromosomes, or, as we have lately termed them, the *Synaptic Gemini*[†] of the first maiotic division. It was indeed the striking peculiarities presented by the forms assumed by these bodies, and the comparative ease with which they can be distinguished from the true chromosomes of pre- and post-maiotic mitoses, which led Professor Farmer[‡] and one of us some years ago to make a list of the different forms we encountered in different animals and plants. We utilised this comparison in establishing what was at that time by no means clear, namely, that in regard to the maiotic divisions we are dealing with an identical process throughout both animals and plants.

In the note referred to and in subsequent publications by other authors, the view has been taken that the adult gemini (heterotype chromosomes) are to be regarded as capable of assuming any of the forms which two flexible rods can take. As for example, when bent round each other; lying parallel to each other; associated together in the form of a cross; joined end to end in the form of a ring; and so on. The rarer forms, such as tetrads, being accounted for by supposing that the four-fold figure results from a thickening or clubbing of the ends of the associated chromosomes thus:— = ∷. In many instances where tetrads are found this is undoubtedly the explanation of their form.

Thus the possible modes of association between two flexible rods, together with the effects of alteration in their form, and the action of the spindle fibres, have hitherto been regarded by ourselves and others as sufficient to account for the great variety of aspect which the maiotic gemini are found to present.

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[*] The title of the paper, as originally communicated and read, was "On the Constancy of Form among the Synaptic Gemini (Heterotype Chromosomes) in Certain Animals."

[†] Moore and Embleton, "On the Synapsis in Amphibia" (preceding paper).

[‡] Farmer and Moore, "On the Essential Similarities existing between the Heterotype Nuclear Divisions in Animals and Plants," 'Anat. Anzeiger,' 1895.

In later years several authors have drawn attention to the obvious difference in size exhibited between one and another of the gemini on the same spindle. And not infrequently one or more of the gemini, under the title micro-chromosome or accessory chromosome, have been supposed to play an individual and important part in matters connected with hereditary transmission and the determination of sex.*

Our attention has latterly been redirected to the exact nature of the first maiotic (heterotype) division, owing to the fact that this form of mitosis has been found to occur during the development of malignant growths in man.†

In 1903, in conjunction with Professor Farmer, we were able, from an extended series of observations, to elaborate a general scheme of the maiotic process which appears to hold good throughout the higher animals and plants.‡ More recently we have dealt in detail over again and extended our observations upon the maiotic process in mammals and amphibia ;§ this revision having become apparently necessary, owing to the mutually divergent accounts of the maiotic process recently published by Strasburger and his pupils in relation to certain plants.||

A re-examination of mammals and amphibia has however confirmed our original standpoint with respect to the maiotic process in these vertebrates, and a similar confirmation has been given for plants in the case of Dictyotaceæ by Lloyd-Williams,¶ Gregory, and others. During the course of these revisions we have been led once more to the questions associated with the forms assumed by the gemini of the first maiotic division, and it appears to us now that the existing conceptions regarding this matter have become inadequate to meet the actual facts of the case. We do not at present profess in any way to have reached a final standpoint in our conceptions of the nature of the different forms of gemini, but the phenomena that have been brought to light are so remarkable in themselves, and from the point of view of theoretical developments so peculiarly attractive, that we

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* For example, see Strasburger, "Typische und Allotypische Kernteilung.," 'Jahrb. f. Wiss. Botanik,' vol. 42 ; O. Rosenberg, *op. cit.*, and others.

† Farmer, Moore, and Walker, "On the Resemblances between the Cells of Malignant Growths and those of Normal Reproductive Tissues," 'Roy. Soc. Proc.,' 1903.

‡ Farmer and Moore, "New Researches concerning the Heterotype Divisions in Animals and Plants," 'Roy. Soc. Proc.,' 1903 ; Farmer and Moore, "On the Maiotic Phase (Reduction Divisions) in Animals and Plants," 'Quart. Journ. Micr. Sci.,' vol. 48.

§ Moore and Embleton, "On the Synapsis in Amphibia" (*loc. cit.*) ; Moore and Walker, "On the Maiotic Phenomena in Mammalia," 'Thomson Yates Reports,' University Press, Liverpool, 1906.

|| Strasburger (*loc. cit.*). See also Overton, Miyake, and Allen, 'Jahrb. f. Wiss. Botanik,' vol. 42.

¶ J. Lloyd-Williams, "Studies in the Dictyotaceæ, I and II," 'Ann. Bot.,' 1904.

desire to publish them in the hope that other cytologists may be able to throw fresh light upon the facts which we have already ascertained.

If the testes of Triton be examined at the time when the first maiotic division is abundant, groups of dividing nuclei may be found presenting the appearance given in fig. 1 (Plate 24). The drawing is an accurate representation of a field of such dividing cells, and upon examination it may be found that the gemini (heterotype chromosomes) lie either upon young spindles or in groups within cells where the nuclear membrane has only lately disappeared.

If in such a field we consider some special form assumed by the gemini, such as the U, or ∝ ; it will be seen that it is generally possible to find this form in any individual cell we like to examine.

So also if we take another conspicuous class of gemini which may be represented thus:—Ð, we again find that this type also can be traced in almost any individual cell we please.

If for the time being we confine our attention to these two forms, and pass from the particular field represented in fig. 1 to a large number of similar fields, the conclusion is quickly forced upon us that the two forms in question are really always present in all first maiotic spindle figures. We soon see in fact, that the instances where one or other is not conspicuous, are due to the particular gemini being turned in such a manner that they become foreshortened in the line of vision, or are obscured by other gemini, either totally or in part.

But besides the two forms of gemini above considered there are in Triton others. Thus we find, as cells in fig. 1 will show, gemini which present the appearance of two rods either lying parallel to one another, or crossed over one another, thus:— = ⋈. Again, there is a form consisting of a bent figure with symmetrical thickenings which form one aspect may be represented thus:—◊. Another form consists of an asymmetrical annulus with unequal sides, thus:—▷. While, lastly, we have a long and evenly thick ring, thus:—O. In this way it will be seen that in the first maiotic division of Triton there are to be found six varieties of gemini, and upon looking into the matter, we are led to conclude that all these six varieties co-exist in every instance of the first maiotic division.

In some cases, however, it is possible to see more than one representative of any particular type in one and the same cell, and upon counting the maximum representation of any type found in a particular element, we find in Triton this number is two. Taking each of the six classes or types of gemini in turn, we find that cells may be found that show two representatives of every one of the six classes.

In Triton the number of the pre-maiotic chromosomes is 24. These in the synapsis unite to form 12 gemini, and consequently we are driven to the conclusion that in the first maiotic spindle figure there exists a pair of gemini belonging to each of the six different types.

The fact that the varieties are constant in the early spindle figure, really in itself precludes the possibility of the different forms having anything to do with the fortuitous manner in which the gemini may become attached to the spindle fibres, and this indication is enforced to the point of proof by the further observation, which can be readily made, that all six varieties of gemini are present in cells before the nuclear membrane has disappeared, that is to say, before the spindle fibres have ever acted on them. In fig. 2 we have a drawing of five cells in three of which the nuclear membrane is not yet ruptured, but in each of these three cells representatives of the different classes of gemini are as clearly to be discerned as they are on the early spindle figures.

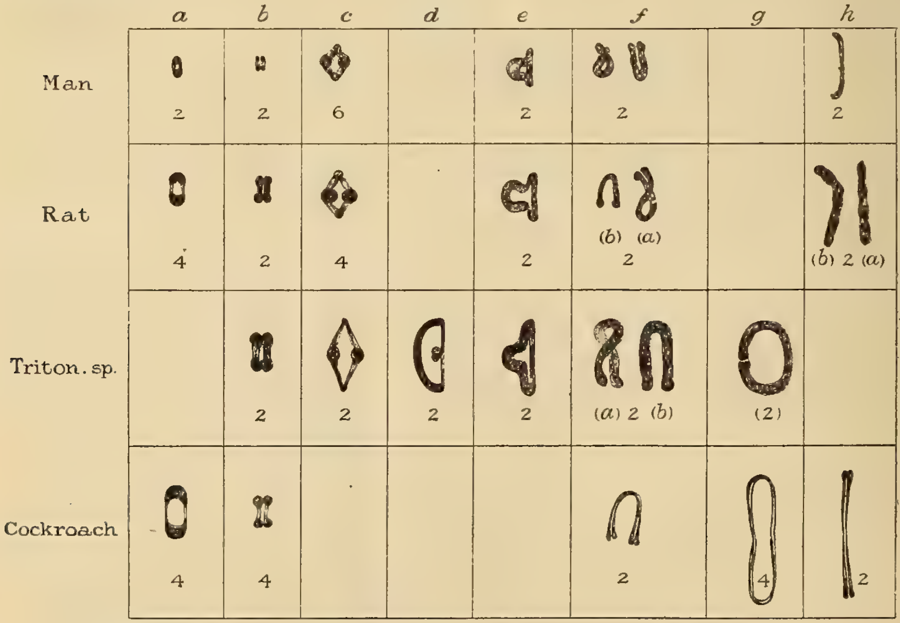

Diagram showing the Forms of Heterolytic Gemini in various Animals.

| | Total number of gemini. | | Total number of gemini. |
|---|---|---|---|
| Man ............... | 16 | Triton, sp. ......... | 12 |
| Rat ............... | 16 | Cockroach ......... | 16 |

Another feature which should be noted is the fact that we do not encounter transitional forms passing from one form of the gemini to another; there is no half-way set of gemini between the forms *a* and *b* given in the table on p. 566, or between *c* and *d*, *e* and *f*, and so on.

The gemini in Triton, as we believe is the case in every other instance of the first maiotic division, are produced by the conjugation of premaiotic (somatic) chromosomes in pairs during the synaptic rest;* and since there are 24 premaiotic chromosomes in the particular instance of Triton, it follows that there must be only four individual chromosomes which can unite with each other to form the two gemini belonging to each of the six types.

The above results, based upon a study of the gemini in the first maiotic divisions in Triton, are interesting in themselves, but they immediately raise the further question as to whether the order here observed is simply a curious instance, or an individual expression of a wider law; on account of this we have studied in a similar manner the first maiotic division in man, rats, and Periplaneta; that is to say, in two more typical vertebrates and a representative arthropod.

In the testes of rats the first maiotic division occurs in groups of cells, and it is by no means difficult to bring under observation in a short time thirty or forty instances of the early spindle and late prophase. In this we have material amply sufficient to arrive at a decision upon the matters with which we are concerned. Fig. 3 is a drawing of a portion of a tubule from the testes of a piebald rat. It shows the outer wall of the tubule with some premaiotic nuclei and three dividing cells belonging to the first maiotic (heterotype) mitosis.

In these it will be seen that the gemini are of very different forms, and that as in Triton the same forms are repeated in different individual cells. Analysis of a large number of similar cells reveals the fact that in this particular example there are again six varieties of gemini (see table, p. 566). In the rat, however, instead of the premaiotic chromosomes being 24 in number as in Triton, there are 32; consequently we shall have to ascertain the relative numbers of the different types. Further it will be seen that the six types present in the rat are not all the same as those catalogued for Triton (table, p. 566). It will indeed be obvious from this that only four of the Amphibian varieties are represented in the mammal; to these four two new forms of gemini are added.

In regard to the type *h* in the table, p. 566, it is obvious that this particular form of the gemini might be regarded as an opened out U, which in this case appears as a straight rod; but although this is so, the fact remains that the types *h* and *f* are present together in rats in such a manner as to suggest that they are really distinct entities.

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* Farmer and Moore, 'Roy. Soc. Proc.,' 1903, *loc. cit.*; Farmer and Moore, 'Quart. Journ. Micr. Sci.,' vol. 48, *loc. cit.*; Moore and Embleton, 'Roy. Soc. Proc.,' 1905, *loc. cit.*; Moore and Walker, 'Thomson Yates Reports,' *loc. cit.*

In rats we have said that the number of the pre-synaptic chromosomes is 32 and the number of gemini is 16. These latter bodies are grouped into six varieties, and consequently the number of each variety in rats must be unequal.

If in rats in a large number of division figures the maximum number of all the six varieties of gemini are counted, as was done in Triton, the results are as follows: *a*—4, *b*—2, *c*—4, *e*—2, *f*—2, *h*—2.*

Upon comparing the above results with similar results in man we find that here the varieties remain the same as in rats, but the relative numbers of these varieties are again changed (see fig. 6, Plate 25, and table, p. 566), the arrangement in man being as follows: *a*—2, *b*—2, *c*—6, *e*—2, *f*—2, *h*—2.

For any one species the numbers of types of gemini, so far as we have gone, appears to be constant, and the same types are retained in the case of fairly remote genera, such as *Homo* and *Mus*; but in these genera the relative numbers of the different kinds of gemini may vary with, or independently of, the number of the premaiotic chromosomes.

Passing from the above vertebrate examples to the old arthropodean type Periplaneta we find, as fig. 5 and the table on p. 566 will show, that here the number of the types of gemini is reduced from six to five.

Upon consideration of the table it will be seen also that three of the amphibian and mammalian types are retained, but no new type is added, and two of the types common to both the other groups are altogether wanting.

In Periplaneta there are 32 premaiotic chromosomes and 16 gemini, so that here, as in the case of man and rats, the number of similar forms must be unequal.

Counting the maximum number of any type in a number of cells, as was done in the former cases, we get the relative number of the five types in each cell as follows: *a*—4, *b*—4, *f*—2, *g*—4, *h*—2.

The possible bearing of the above observations upon the various existing theories of hereditary transmission, and especially in relation to the Mendelian hypothesis, will be obvious enough; but we feel a great reluctance at the present time in any way to augment the obscuration of the facts by putting forward crude theoretical anticipations.

What appears to us of first importance is the recognition of the actual existence of permanent structural types in the gemini of different forms. Secondly, it would appear that in any particular form the number of gemini of each type have a constant numerical relationship to each other. Thirdly, so far as the investigation has at present gone, certain types of gemini appear to be common to all the widely sundered forms examined. Still further, it will be seen that the number of different types of gemini is less in the oldest evolutionary form Periplaneta.

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* It is an interesting and important fact that the number of premaiotic (somatic) chromosomes is not the same in rats as in mice. In the latter the number is 24.

Whether this last indication will be found to hold good is a matter upon which it would at present be useless to speculate; but the fact itself opens up a line of future inquiry which is certainly full of possibilities.

It seems to us, moreover, that it should be emphasised that both in regard to the permanent types of gemini and their numerical relationships, as well as with respect to the numerical constancy in the chromosomes themselves and their periodical reductions, we are face to face with constant arrangements in the parts of the unit of living substance (the cell) which seem to underlie and to be quite independent of those external interactions that are supposed to have helped to build the grosser features of living things.

With regard to the different types of gemini, it should further be pointed out, that the existence of these types implies substantive differences between the chromosomes that can unite to form the different kinds. It must be remembered that each of the gemini arises through an association of optically similar premaiotic chromosomes, but that at the time the nuclear membrane is about to disappear these associations have assumed different forms. They cannot do this unless they are of a different nature. The fact that there exist in those nuclei which we have examined groups of similar gemini shows that there must be sets of premaiotic chromosomes which in the synapsis can conjugate with each other, but not with the remaining individuals.

The present position may be in part summed up as follows:—In the fertilised egg the paternal and maternal chromosomes divide independently on the spindle of the first segmentation figure. And they go on dividing in a similarly independent manner throughout the soma, and during the premaiotic history of the reproductive elements themselves. In the synapsis which ushers in the maiotic phase the chromosomes unite in pairs, and in those cases we have as yet examined only certain individual chromosomes are capable of uniting with one another to form differing group of gemini; in each of these groups the number of gemini is more than one, and it varies in the different species hitherto observed.

Thus whether the conjugation of the chromosomes in the synapsis is really the final consummation, after many generations long delayed, of the copulatory intentions of the paternal and maternal elements, is a matter upon which there is as yet no actually conclusive evidence.

## DESCRIPTION OF PLATES.

### Plate 24.

Fig. 1.—Groups of cells from the testis of a Triton. They are all in phases of the first maiotic (heterotype) division, and similar forms of gemini (heterotype chromosomes) are to be seen in numbers of different cells, as at *b*, *c*, *d*, *g*, *f*.

Fig. 2.—Cells from the testis of a Triton, all in the late prophase of the first maiotic division. In three of the cells the nuclear membrane is still present; but the gemini have already assumed the same forms as those represented in fig. 1, *b*, *c*, *d*, *g*, *f*.

Fig. 3.—Cells from the testis of a rat, showing similar gemini in the different cells, *a*, *c*, *e*, *f*, *h*.

### Plate 25.

Fig. 4.—Cell from testis of a rat. Early phase of first maiotic spindle, showing pairs of different gemini, *a*, *b*, *c*, *d*, *e*.

Fig. 5.—Group cells from the testis of Periplaneta, showing similar gemini in each of the three different cells, *a*, *b*, *f*, *g*, *h*.

Fig. 6.—Cells from the testis of man, showing similar gemini in the three cells at *a*, *c*, *e*, *h*.

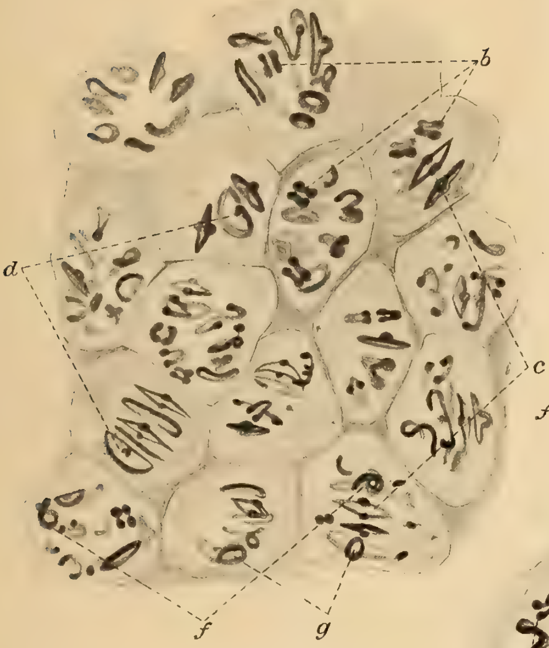
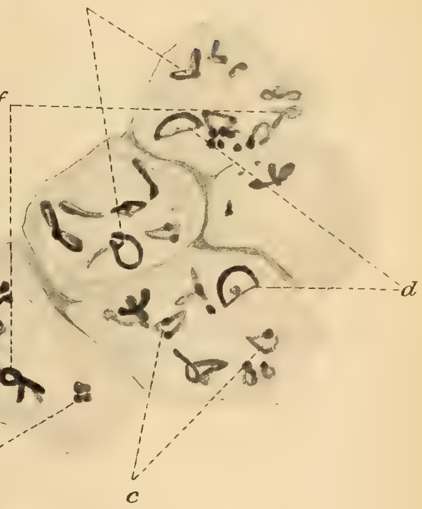


FIG. I.

FIG. 2.



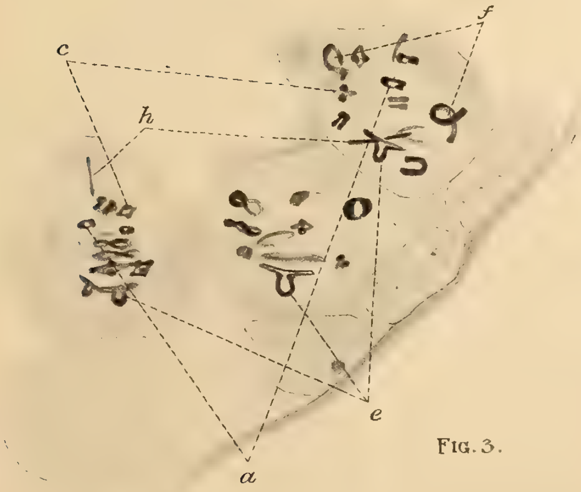


FIG. 3.

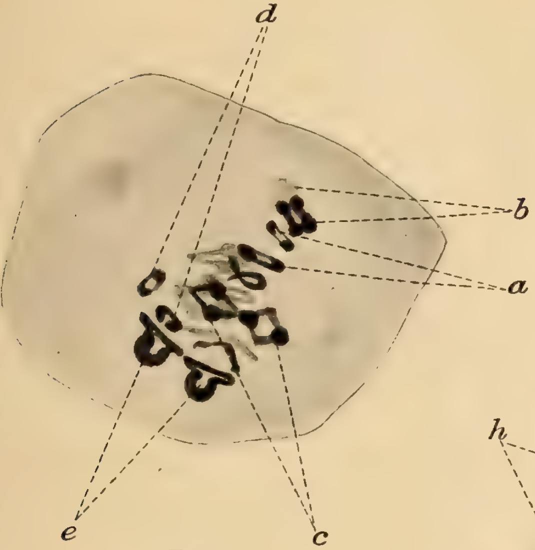
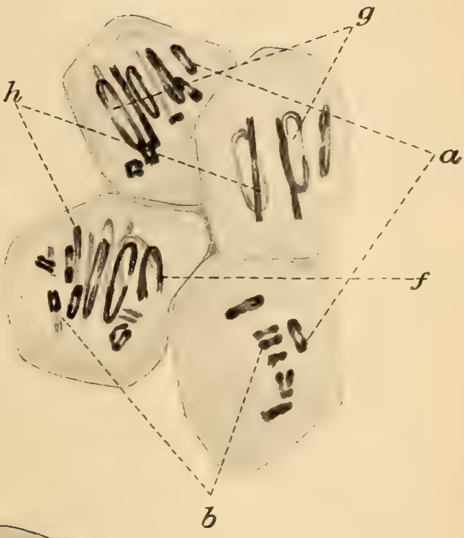


FIG. 4.

FIG. 5.



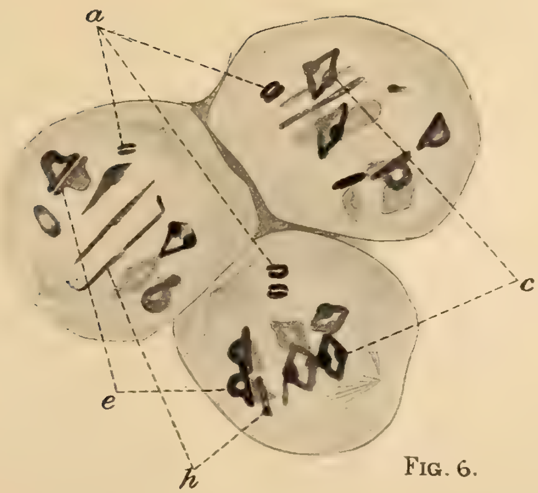


FIG. 6.

# *The Action of Pituitary Extracts upon the Kidney.*

By E. A. Schäfer, F.R.S., and P. T. Herring.

(Received May 2,—Read May 3, 1906.)

(From the Physiological Laboratory of the University of Edinburgh.)

(Abstract.)

Intravenous injections of saline extract of the infundibular part of the pituitary body produce dilatation of kidney vessels accompanied by increased flow of urine; *i.e.*, the extract has a diuretic action.*

With the first injection this result is accompanied by the rise of blood-pressure and contraction of systemic arteries described originally by Oliver and Schäfer,† and since confirmed by various observers.

With subsequent injections (if the first injection were not too small in amount) administered within a certain interval of time after the first one, the diuresis is usually attended not by a rise of blood-pressure,‡ but by a fall (depressor effect).§ This fact furnishes evidence that the diuresis is independent of the effects upon blood-pressure and leads one to suppose that it is produced by a special constituent of the extract.

This conjecture is confirmed by the result of treating the extract with a peptic digestive fluid or with hydrogen peroxide. These agents tend to abolish the rise of blood-pressure which is produced by a first injection, but leave the diuretic effect of the extract unaltered. Reducing agents and the action of tryptic digestive fluid leave all the active constituents of the extract apparently unaffected.

The diuretic as well as the pressor and depressor constituents of the extract are not destroyed by boiling. They dialyse through parchment paper. They are insoluble in absolute alcohol and ether.

Occasionally, especially with large doses of the extract, the diuretic effect fails to show itself. This appears to be due to the kidney vessels participating in the general vascular constriction which is caused by the extract. More often such constriction of renal vessels is only temporary, and gives place to dilatation with free flow of urine.

Hypodermic injections produce effects similar to those caused by intra-

---

* See Schäfer and Magnus, 'Physiol. Soc. Proc.,' p. ix, in 'Journ. Physiol.,' vol. 27, 1901.

† 'Journ. Physiol.,' 1895, vol. 18.

‡ W. H. Howell, 'Journ. Exp. Medicine,' vol. 3, 1898.

§ Schäfer and Vincent, 'Journ. Physiol.,' 1899, vol. 25.

venous injection, but of a far less marked character and coming on only gradually and after a long interval. Introduction of the extract into the stomach is followed by even less noticeable effects. It is inferred that the active constituents are not absorbed by the gastric mucous membrane with sufficient rapidity to produce the usual symptoms.

Intravenous injections of extracts from the anterior or epithelial lobe of the pituitary body do not produce diuresis: these extracts exhibit no physiological activity.

It is concluded that the infundibular part of the gland produces an internal secretion which passes into the blood and which, both indirectly owing to its general action upon the vascular system and directly by its special action on the renal vessels and renal epithelium, assists in promoting and regulating the secretion of urine; in other words, the internal secretion of the gland is ancillary to the renal functions.

Finally the relations of the pituitary body to the functions of the suprarenal and thyroid glands and to the production of acromegaly are briefly discussed.

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